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13. ABSTRACT ***(Maximum 200 Words)***

Isolation and cloning of a conserved cdc25-interacting protein, Pin1, provides the opportunity to explore the role of this binding partner in cooperating with cdc25 to regulate cell cycle transitions. Pin1 is a peptidyl prolyl *cis-trans* isomerase; its yeast homologue is essential for proper execution of mitosis and has implied roles in regulating transcription through its effects on chromatin structure and RNA processing. Presented here are data that demonstrate a role for Pin1 in negatively regulating the mitotic entry in *Xenopus laevis*. Without catalytically active Pin1, egg extracts initiate mitosis prematurely and do not activate the G_2 checkpoint that arrests the cell cycle when DNA replication is stalled. Because it selectively binds to and catalyzes isomerization of prolines preceded by phosphorylated serine or threonine, Pin1 may exert its inhibitory influence on mitotic entry by affecting phosphoprotein structure. Together with the work of other groups, this study suggests a role for Pin1 in controlling the timing of mitotic entry by isomerizing phospho-S/T-proline subsequences and thereby influencing the abilities of these motifs to be substrates for the mitotic phosphatase, cdc25.

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Introduction

Dividing cells faithfully transmit genetic information through a highly regulated process referred to as the cell cycle. Ordered progression of signaling and structural events ensures accurate reproduction of the genome and its segregation into daughter cells. Regulatory mechanisms known as checkpoints compartmentalize this division process into biochemically discrete units, the beginning of one depending upon successful completion of another, and thus comprise pathways that permit or prohibit cell cycle progression (Hartwell and Weinert, 1989). A G_2 checkpoint governs the transition from interphase to mitosis, postponing the process when DNA is damaged or incompletely replicated (Kaufmann, 1995), a situation that, without repair, would lead to fatal mitotic catastrophe or endoreduplication. Many conserved molecular components participate in the regulatory and surveillance pathways that affect G_2/M progression, and intricate crosstalk provides tight control of this cell cycle transition. Cdc2 kinase is centrally responsible for mitotic initiation (King *et al.*, 1994; Nurse, 1990). It phosphorylates targets required to orchestrate the dynamic processes characteristic of mitotic entry, including nuclear envelope breakdown, chromatin condensation, microtubule destabilization, and spindle formation (Nigg, 1993). As the point of biochemical convergence governing G_2/M progression, Cdc2 functions as a molecular signal integrator controlled by several layers of regulation (Morgan, 1997). Temporal and spatial coordination of its activation ensures that mitosis-promoting activity occurs in the correct place, at the correct time, and only under the correct circumstances. Combinatorial effects of phosphate incorporation by Cdc2 and its periodically available cyclin B1 subunit (Meijer *et al.*, 1989), dictate the activity state and subcellular localization of the complex. In this respect, the Cdc2/cyclin B1 complex provides the focal point for G_2/M regulation by kinases and phosphatases, and its rapid and complete activation stimulates mitotic entry. The phosphatase, cdc25, is responsible for the final biochemical event in initating mitosis: removal of inhibitory phosphates on cdc2 (Coleman and Dunphy, 1994; Gabrielli *et al.*, 1992; Kuang *et al.*, 1994; Russell and Nurse, 1986). Overexpression of cdc25 in breast cancers is well documented (see original grant application) and correlates with disease progression. The aim of this research as proposed was to investigate the mechanism by which cdc25 contributes to breast cancer progression.

Body: Background

Following initial collaborative studies of cdc25 regulation, the bulk of the research efforts funded by this grant have gone toward the study of a cdc25-interacting protein, Pin1. This protein was initially cloned from *A. nidulans* (*pinA*) and human (*pin1*) libraries in yeast-two-hybrid screens by virtue of its interaction with another G_2/M-promoting kinase, NIMA (Crenshaw *et al.*, 1998; Lu *et al.*, 1996). Because overexpression of active NIMA alone is deleterious in yeast, the identification of Pin1 these screens indicates that it suppresses the lethality of overexpressed NIMA protein and hints that it might functionally oppose NIMA. The deduced amino acid sequences contain regions of homology to a family of prokaryotic peptidyl-prolyl *cis-trans* isomerases (PPIases), the parvulins (Rudd *et al.*, 1995), and to the WW protein interaction domain recognized for its binding affinity for proline-containing sequences (Sudol, 1996). *ess1/ptf1* and *dodo* genes of *S. cerevisiae* (Hanes *et al.*, 1989; Hani *et al.*, 1995) and *Drosophila melanogaster* (Maleszka *et al.*, 1996), respectively, were cloned previously in unrelated studies and share these domain features with both *Aspergillus* PinA and human Pin1. The abilities of both *dodo* and *pin1* genes to rescue the lethality of *ess1* deletion (Lu *et al.*, 1996; Maleszka *et al.*, 1996) establishes functional homology among these family members. Interestingly, biochemical analyses highlight an unexpected phosphorylation-specificity in proline sequence recognition by both of Pin1's modular domains. Its WW domain binds preferentially to phospho-serine/threonine-proline motifs (Landrieu *et al.*, 2001; Lu *et al.*, 1999b; Verdecia *et al.*, 2000), and while Pin1 catalyzes prolyl isomerization as predicted, it is unlike its relatively non-selective

immunophilin cousins in that Pin1 shows appreciable catalytic activity only toward prolines preceded by phospho-serine/threonine but not phospho-tyrosine (Hani *et al.*, 1999; Lu *et al.*, 1999b; Ranganathan *et al.*, 1997; Yaffe *et al.*, 1997). Thus, it seems that Pin1 is an evolutionarily conserved protein that is structurally and enzymatically poised to interact with phosphorylated proteins.

Indeed, Pin1's ability to bind phospho-proteins in a number of systems has been documented (Crenshaw *et al.*, 1998; Gerez *et al.*, 2000; Hsu *et al.*, 2001; Liu *et al.*, 2001; Lu *et al.*, 1999a; Pathan *et al.*, 2001; Shen *et al.*, 1998). Studies using recombinant Pin1 as an affinity reagent demonstrate that its interaction with most proteins is dependent on their phosphorylation states. For example, Pin1 binds an extraordinary number of mitotic phospho-proteins including many recognized by the monoclonal antibody, MPM-2 (Crenshaw *et al.*, 1998; Shen *et al.*, 1998), which recognizes an epitope including phosphorylated serine/threonine-proline residues (Davis *et al.*, 1983; Ding *et al.*, 1997; Kuang *et al.*, 1989). Depletion of Pin1-binding partner(s) from *Xenopus* cycling extracts during interphase (using tagged, immobilized *A. nidulans* PinA protein) causes a G_2 arrest, while similar depletion of M-phase extracts causes a reduction in Cdc2's histone H1 kinase activity without directly removing the Cdc2/cyclin B1 complex (Crenshaw *et al.*, 1998). These experiments demonstrate that Pin1 binding proteins are important for both the initiation and maintenance of Cdc2 activation in *Xenopus* extracts.

Among the myriad MPM-2 reactive mitotic phospho-proteins depleted in these experiments is Cdc25. Pin1 interacts preferentially with hyperphosphorylated Cdc25, and *in vitro*-translated phosphatase can be converted into a Pin1-binding protein by the addition of active Cdc2/cyclin B1 (Crenshaw *et al.*, 1998). While this observation provided the initial motivation for using Pin1 to further our understanding of cdc25, recently published results provide mechanistic insights into the nature of the Pin1/cdc25 interaction that have helped in our interpretation of our biological results. Namely, incubation of phosphorylated Cdc25 with sub-stoichiometric (catalytic) concentrations of Pin1 changes the pattern of protease digestion, indicating that Pin1 not only binds, but also alters the conformation of Cdc25 (Stukenberg and Kirschner, 2001). Further evidence for Cdc25 isomerization by Pin1 comes from the observation that *in vitro* phosphorylated Cdc25 is recognized by the MPM-2 antibody only after incubation with Pin1 (Stukenberg and Kirschner, 2001). As yet, it is not clear what consequence this structural modification has on Cdc25's catalytic activity, but it does enhance the rate of Cdc25 dephosphorylation by protein phosphatase 2A (PP2A)(Zhou *et al.*, 2000b). PP2A preferentially dephosphorylates *trans* isomers of the phospho-serine/threonine-proline motif; this confirmation is favored in the presence of catalytically active Pin1. In addition, overexpression of Pin1 and PP2A are each able to suppress growth arrest phenotypes in yeast strains bearing conditional genetic lesions in the reciprocal gene. These studies imply that, at least for PP2A and MPM-2, substrate or epitope recognition, respectively, requires a specific isomer of the prolyl bond adjacent to the phosphorylated side chain. It is intriguing to speculate that modulation of protein function following phosphorylation may be, in some cases, the result of phosphate-directed prolyl isomerization. Pin1's catalytic preference for phosphorylated sequences, which makes the connection between protein phosphorylation and changes in protein conformation, is unique among eukaryotic PPIases.

Perhaps this substrate selectivity explains the requirement of the gene encoding the yeast Pin1 homologue, *ess1*, for viability (Hanes *et al.*, 1989; Hani *et al.*, 1995). In contrast, all twelve yeast immunophilin genes can be deleted, even in combination, without lethality (Dolinski *et al.*, 1997). Inactivation of ESS1 causes terminal mitotic arrest and nuclear fragmentation (Hanes *et al.*, 1989; Wu *et al.*, 2000), suggesting a function in mitotic exit. Curiously, neither *dodo* from *D. melanogaster* nor the mouse Pin1 homologue is required for viability, possibly due to genetic redundancy (Campbell *et al.*, 1997; Uchida *et al.*, 1999). There is limited evidence that Pin1 null mouse embryonic fibroblasts are defective in their ability to re-enter the proliferative cycle following release from a serum-

starvation-induced G_0 arrest (Fujimori *et al.*, 1999). Recently researchers have reported a role for *dodo* in influencing mitogen-activated protein kinase (MAPK)-dependent transcriptional regulation. Apparently, phosphorylation of the transcription factor, CF2, on serine/threonine-proline sites by MAPK causes its degradation in a process requiring the *dodo* gene product (Hsu *et al.*, 2001). These functions in metazoans still do not explain the failure of *ess1* mutant yeast strains during mitosis.

Body: Results and Discussion

As this project was initiated, the cdc25 activation was known to involve phosphorylation of its N-terminus (Izumi *et al.*, 1992). The direct effect of this modification was not well understood, although it was recognized that, following some minimal action of signal-initiating kinase, much of the hyperphosphorylation of Cdc25 observed upon mitotic entry is attributable to Cdc2 itself, which phosphorylates Cdc25 on multiple sites, stimulating its phosphatase activity, and thereby leads to activation of more Cdc2/cyclin B1 (Izumi *et al.*, 1992). Thus, a catalytic positive feedback loop amplifies the initiating Cdc25 signal to coordinate the complete and abrupt activation of Cdc2 (Hoffmann *et al.*, 1993). The identity of the "initiating kinase" is still debated, but recent insight, in part the result of research supported by this grant, has expanded our understanding of cdc25 regulation to include a second, but arguably more decisive, level of control of mitotic initiation.

Throughout interphase, cdc25 is physically sequestered to prevent premature functional interaction with and activation of Cdc2/cyclin B1. We and others have shown that phosphorylation of Cdc25 on a single serine residue (216 in human, 287 in *Xenopus laevis*) by Chk1 and/or Cds1 kinases (Furnari *et al.*, 1999), confers to it the ability to bind cytoplasmic 14-3-3 proteins (Dalal *et al.*, 1999; Graves *et al.*, 2001; Kumagai and Dunphy, 1999; Peng *et al.*, 1997; Yang *et al.*, 1999), thereby preventing Cdc25 accumulation in the nucleus until G_2/M. Furthermore, we have provided evidence that, even while phosphorylated at this residue cdc25, retains its basal catalytic activity (Yang *et al.*, 1999). Thus, it seems feasible that inappropriate activation of Cdc2/cyclin B1 would follow cdc25 overexpression if it reached levels sufficient to saturate the available 14-3-3. Consequently, early mitotic entry would be expected to contribute genomic instability that is often associated with development and progression of cancers.

The importance of suppressing cdc25's function in maintenance of genomic stability is underscored by the circuitry of endogenous responses to genotoxic lesions. When the DNA is damaged or replication is stalled, the abnormal structures trigger a cascade of events to elicit a temporary cell cycle arrest known as a checkpoint. Cdc25 is the target of the G_2 checkpoint kinase cascade, the most proximal component of which is ataxia-telangiectasia-mutated kinase (ATM) and/or the ATM-related kinase, ATR (Hekmat-Nejad *et al.*, 2000; Hoekstra, 1997; Shiloh, 2001). In response to genotoxic stress, ATM/ATR activation leads to increased activity of checkpoint kinases Chk1 and Cds1 (Kumagai *et al.*, 1998; Zeng *et al.*, 1998; Zhou *et al.*, 2000a), thereby exploiting existing regulatory machinery to enhance phosphorylation of Cdc25 serine 216 and prolong its cytoplasmic sequestration (Boddy *et al.*, 1998; Forbes *et al.*, 1998; Furnari *et al.*, 1999; Lopez-Girona *et al.*, 1999; Peng *et al.*, 1997; Sanchez *et al.*, 1997; Yang *et al.*, 1999).

As the research funded by this grant began, Pin1 had been implicated in cell cycle regulation primarily in overexpression studies, and its ability to associate with known mitotic regulators, including cdc25 had just been recognized. Whether this interaction occurred *in vivo*, and what role Pin1 played in cell cycle regulation if any, was not known. The model system selected for addressing this question was that of *Xenopus laevis* egg extracts. This system has been used successfully to identify and characterize many of the molecules now recognized as universal eukaryotic cell cycle regulators. Specifically relevant to studies of endogenous Pin1 function, however, is the feasibility of removing the protein directly from the extract and assessing the immediate effects on isolated cell

cycle transitions.

Xenopus eggs that are arrested at metaphase of meiosis II are laid in response to hormonal cues. Fertilization mobilizes calcium and spurs the egg's progression out of M phase and into a rapid series of cell divisions. The early embryo increases its cell numbers without significant accumulation of biomass by oscillating between S and M phases. To do this, the egg must already be stocked with enough cytoplasmic components for all its resulting daughter cells. Thus, *Xenopus* eggs are biochemical warehouses of cell cycle components. In addition to stockpiling proteins and organelles, eggs also prepare for this dedicated proliferative phase by preparing ahead of time all the mRNA it will need for these cycles. This investment on the part of the oocyte allows the zygote to bypass any need for transcription, and even its translational efforts are largely dedicated to synthesizing cyclin B1 for each oscillatory transition from S phase into M phase. In fact, extracts of *Xenopus* eggs can be treated with inhibitors of transcription with no apparent deleterious effects on cell cycle progression, and the translation inhibitor, cycloheximide, is often used to synchronize extracts that can subsequently be prompted to enter mitosis by addition of recombinant cyclin B1. These biological characteristics of the egg extract system have made it especially amenable for use in the study of isolated cell cycle transitions.

Before Pin1's role in the *Xenopus* cell cycle could be ascertained, it was first necessary to clone the *Xenopus* version of the gene, confirm its homology to other members of the Pin1 family, and generate reagents with which to remove the protein from extracts. Considering the known existence of yeast, *Aspergillus*, fruit fly and human Pin1 homologues, including the demonstrated abilities of fly and human enzymes to complement *ess1* deletion in yeast, there was good reason to suspect the existence of a closely-related *Xenopus* Pin1. The human Pin1 coding region (Lu *et al.*, 1996) was used to probe plaque lifts, obtained by bacterial infection with a lambda phage *Xenopus* gastrula cDNA, under low-stringency hybridization conditions. Three clones were identified among roughly 5×10^5 plaques screened. Each of these contains an identical open reading frame that we designate xPin1 (Figure 1A). The predicted polypeptide sequence shares 89% identity with human Pin1 and greater than 45% identity with each of the eukaryotic parvulins over its full length of 159 residues (Figure 1B). *In vitro* transcription directly from the cloned cDNA yields mRNA (Figure 2Aa) that, when translated *in vitro*, encodes an 18 kDa protein as predicted (Figure 2Ab).

The xPin1 cDNA expressed as an N-terminally tagged glutathione-S-transferase fusion protein (GST-xPin1) in bacteria is readily purified to homogeneity, and xPin1 is efficiently cleaved from the GST moiety (Figure 2B). Recombinant xPin1 conjugated to hemocyanin was used as antigen to generate polyclonal antisera. Sera from two immunized rabbits recognize a single protein of 18 kDa in *Xenopus* egg extracts (Figure 3A and data not shown). The concentration of Pin1 in egg extracts is roughly 20 ng/μL or approximately 1 μM, and this does not change throughout the cell cycle (Figure 3B).

Because *Xenopus* Pin1 shares such a high proportion of its primary amino acid sequence with mammalian versions of the protein, the antisera generated against it recognize Pin1 homologues in mouse and human tissue extracts. To delineate the expression pattern of Pin1 in mammals, extracts prepared from whole organs of six-week-old mice were subjected to immunoblot analysis. As shown in Figure 4Aa, although low levels of expression are detectable in most adult mouse tissues, testis expresses Pin1 to high levels, while cerebellum, kidney, and stomach show moderate expression. By comparison, Pin1 is expressed ubiquitously to high levels in neonatal mice (Figure 4Ab), suggesting that expression of the protein is down-regulated at some time during post-natal development in most tissues that do not continue to proliferate throughout the life of the organism.

From whole tissue extract containing a heterogeneous mixture of cell types, however, it isn't possible to determine whether Pin1 is expressed in proliferating cells. To specify the Pin1-expressing

cells within the testis, affinity-purified anti-xPin1 antibody was used in immunohistochemical analysis of fixed tissue. Panels a, c, and e of Figure 5 show testis stained for Pin1 (brown) and counterstained with methyl green (DNA, blue/green). Pachytene spermatocytes near the periphery of each tubule, which are executing meiosis I, are most intensely stained. That this staining is specific to Pin1 and not the result of antibody cross-reactivity is confirmed by parallel staining of testis from a $Pin1^{-/-}$ mouse (Figure 5 panels b, d, and f). Thus, it appears that Pin1 is expressed in cells undergoing meiosis.

To examine more directly whether actively cycling cells express Pin1, human biopsy samples were subjected to immunoblot analysis. Paired biopsy samples of tumor (T) and adjacent normal (N) tissue from patients with colon (a), kidney (b), liver (c), and lung (d) cancers were compared (Figure 6). As seen in adult mouse, both kidney and gastro-intestinal tissues express Pin1 to high levels, and there is no observable difference between normal and tumor samples. Expression levels are lower in normal liver, but, again, the tumor samples are comparable to normal samples in expression level. In contrast, each of the five lung tumors overexpresses Pin1 relative to the low levels of expression apparent in adjacent normal tissue.

The *Xenopus* extract system provides the opportunity for directly examining the effects of Pin1 overexpression. When recombinant xPin1 is added to cycling extracts during interphase, it delays the entry into mitosis in a concentration-dependent manner. Histone H1 kinase activity levels in the extract indicating that Cdc2/cyclin B is active one hour after the addition of GST (Figure 7Aa) but not until two hours after the addition of and equivalent concentration of xPin1 (b). The peak of histone H1 kinase activity is accompanied by chromatin condensation and nuclear envelope breakdown (Figure 7B). Figure 7C represents the mitotic index, quantified by fluorescence microscopy, of extracts supplemented with various concentrations of xPin1 ranging from 1 X endogenous (GST, dotted line) to 100 X endogenous (2 μg/μL). While five-fold overexpression of Pin1 does not influence the duration of interphase, G_2 is prolonged by the addition of both ten-and twenty-fold endogenous levels and mitotic entry is postponed indefinitely by addition of 100-fold excess xPin1. This phenomenon is observed only with the cleaved protein and not with the GST-xPin1 fusion protein (data not shown). These observations are consistent with those previously observed using the *Aspergillus* PinA in cycling *Xenopus* extracts (Crenshaw *et al.*, 1998), although as might be anticipated, the heterologous enzyme is considerably less potent in side-by-side experiments (data not shown).

Xenopus Pin1 can be used as an affinity reagent to removed Cdc25 from cell extract. It binds the mitotic hyperphosphorylated form preferentially as shown in Figure 8Aa. This observation further establishes the similarity of *Xenopus* Pin1 with human and *Aspergillus* versions and provides a means of testing the biochemical requirements for association of these two proteins. Mutation of the two tryptophan residues that define Pin1's WW domain completely abrogates Cdc25 binding, while the catalytically inactive version of the protein, $xPin1^{C109A}$, appears to bind Cdc25 slightly more avidly than wild type Pin1. In addition to demonstrating that the active site mutant's structure is not compromised, this observation suggests that the interaction between the two enzymes might be stabilized by the catalytic inability of the C109A mutant. Similar enhancement of binding is observed when this *Xenopus* mutant protein is used in *in vitro* binding experiments with purified *Aspergillus* NIMA (data not shown).

Because of Pin1's demonstrated preference for binding and catalysis of phospho-serine/threonine-proline sequences, the most attractive candidate binding sites in Cdc25 are the Cdc2 sites that have been shown to be responsible for the bulk of Cdc25's mitotic phosphorylation and activation (Izumi and Maller, 1993). Each of these sites was mutated in the context of the wild type protein, and the abilities of the *in vitro* transcribed and translated missense mutants, which had been incubated in *Xenopus* M extract for phosphorylation, to bind Pin1 were assessed. No single mutation

significantly diminishes the binding of the phosphatase to Pin1 (Figure 8B). In contrast, the majority of binding is abrogated by mutation of the three N-terminal threonines together, and additional mutation of serines 205 and 285 does not further reduce Pin1 binding. This suggests that multiple sites contribute to make phosphorylated Cdc25 a Pin1-binding protein, although it does not distinguish whether the identity of the phosphorylated sites is important.

The impact on the cell cycle of removing Pin1-associated proteins, including Cdc25, was determined using *Xenopus* extracts. GST or GST-xPin1 binding proteins were depleted from interphase and mitotic extracts using GSH-sepharose-linked recombinant proteins. Histone H1 kinase activity was assessed after room temperature incubation of extracts with or without the addition of exogenous Δcyclin B1, xPin1, or both. Results indicate that either the bulk of H1 kinases are removed among the Pin1 binding proteins, or the treatment inhibits H1 kinase activity indirectly, perhaps by removing upstream activators thereby favoring the inhibition of mitotic H1 kinase activity (circles). When Pin1 binding proteins are depleted from interphase extracts, the responsiveness of the extract to cyclin B1 is diminished by co-addition of exogenous xPin1 (stars). This suggests that the extract is sensitized to the negative regulatory affects of Pin1 on mitotic entry.

Initial characterization of *Xenopus* Pin1 indicated the protein is abundantly expressed throughout the cell cycle. There is a positive correlation between mammalian Pin1 expression and the proliferative status of cells and tissues. The induced expression of Pin1 in human lung cancers could contribute to or be a byproduct of neoplastic transformation. While this study documents differential Pin1 expression between normal and tumor tissue only in lung cancers but not in liver, colon or kidney samples, it is noteworthy that all the tumors examined express Pin1 to at least moderate levels, and low levels of expression in normal lung samples distinguished lung from the other organs. Unfortunately, the specific lesion(s) leading to neoplasia in these lung cancers are not known, so this interesting observation does not offer insight into the potential causes or effects of Pin1 overexpression.

It would be informative to know, for example, what the status of cyclin D1 expression levels are in the lung samples. A recent evaluation of human breast cancer cell lines and tissue samples demonstrates that Pin1 expression levels correlate with tumor grade and cyclin D1 expression levels (Wulf *et al.*, 2001), and in cells overexpressing BRCA1, Pin1 is one of the most potently suppressed genes (MacLachlan *et al.*, 2000). Transcription of a cyclin D1 promoter-driven reporter gene is activated by Pin1, and Pin1 overexpression causes an increase in the levels of cyclin D1 RNA in breast cancer cell lines (Wulf *et al.*, 2001). The effect is not a result of general transcription stimulation but seems to occur by enhancing effects of Pin1 on the transactivating activity of phosphorylated c-Jun (Wulf *et al.*, 2001). Thus, it seems that yet another aspect of transcriptional regulation is influenced by Pin1, adding to its previously demonstrated genetic and physical interactions with HDAC components and RNA Pol II CTD (Arevalo-Rodriguez *et al.*, 2000; Morris *et al.*, 1999; Myers *et al.*, 2001; Wu *et al.*, 2000). Cyclin D1 expression is commonly upregulated in breast cancers (Bartkova *et al.*, 1994; Gillett *et al.*, 1994), and despite its reputation as an oncogene, histological analyses and epidemiological studies indicate that overexpression of cyclin D1 is associated with positive prognosis and responsiveness of breast cancers to endocrine therapy (Barnes and Gillett, 1998; Gillett *et al.*, 1996). This apparent contradiction might be due to cdk4/cyclin D1-dependent transcriptional activation of cell cycle inhibitors such as p27 (Barnes and Gillett, 1998). Thus, Pin1 upregulation may oppose neoplastic progression by inducing cyclin D1 under circumstances in which the cyclin promotes differentiation rather than proliferation.

The data in *Xenopus* support the developing and complementary view in the field that Pin1 might suppress cell proliferation as a negative regulator of mitotic entry (Crenshaw *et al.*, 1998; Lu *et al.*, 1996; Shen *et al.*, 1998). Addition of Pin1 to interphase extracts stalls or disables entry into mitosis. Pin1 binding to known G_2/M regulators, including phosphorylated Cdc25, provides a

potential target for its theoretical action. Finally, the observation that depletion of Pin1 binding proteins from M phase extracts results in reduction of histone H1 kinase activity to almost interphase levels is tantalizing despite the obviously non-physiologic experimental design. The apparent mitotic arrest that is the result of *ess1* deletion suggests a role for Pin1 in mitotic exit, but in theory this could be a secondary consequence of inappropriate mitotic entry. To ascertain the nature of Pin1's cell cycle function, the *Xenopus* extract system was exploited to examine the consequences of Pin1 depletion.

Extracts generated in the presence of calcium chelators remain cytostatic factor-arrested (CSF) until the addition of exogenous calcium allows progression into anaphase. Therefore, CSF extracts, once prepared can be subjected to various biochemical manipulations after which the ability to exit M phase can be assessed. On the other hand, lysis of eggs in the presence of calcium renders the resulting extract interphase in nature, and, again, manipulations can be performed prior to evaluating the subsequent cell cycle transition, in this case mitotic entry.

This system for studying cell cycle regulation is especially suited to assessment of Pin1's cell cycle function for several reasons. The gene's essential nature in yeast, in combination with the protein's stability, confound attempts to dissect cell cycle function genetically, and as the studies were begun conditional mutants allowing rapid inactivation of Pin1 protein had not been characterized. Even with the availability of such mutants, however, the *Xenopus* system provides a setting in which to assess Pin1's cell cycle function independently of any effects that might result indirectly from its effects on transcriptional regulation and RNA processing.

The mitotic arrest observed in the *ess1* mutant yeast strain suggests a function for Pin1 in mitotic exit. To test this directly, CSF extracts were used to examine the consequences of removal of Pin1 on mitotic exit and DNA replication. When CSF extracts are immunodepleted of Pin1(Figure 9A), Pin1-depleted and mock-depleted extracts are equally capable of exiting M phase (Figure 9B). Furthermore, Pin1-depletion has no effect on DNA replication (Figure 9C). Thus, in this cell-free system, Pin1 appears not to be required for either S phase or the M/G_1 transition.

Alternatively, the mitotic arrest of yeast could be the consequence of a premature mitotic entry triggered in the absence of Pin1. To examine the effects of Pin1 depletion on the isolated G_2/M transition, cycling or interphase extracts were depleted of Pin1 and subsequently induced to enter M phase either by enabling intrinsic oscillations or by addition of exogenous cyclin B1 protein, respectively. Microscopic examination of nuclei, coupled with measurement of Cdc2-cyclin B1-catalyzed histone H1 phosphorylation, revealed that both types of extract, when depleted of Pin1, enter mitosis more rapidly than do control extracts (Figure 10). Although the absolute timing of mitotic entry varies from extract to extract, removal of Pin1 consistently accelerates the transition into M phase.

The possibility exists that the premature mitosis in Pin1-depleted extract is due to the failure of a negative regulatory influence at the G_2/M transition. The duration of interphase in *Xenopus* extracts can be prolonged by supplementing extracts with high concentrations of sperm chromatin, which increases the time required for DNA synthesis (Dasso and Newport, 1990). The presence of unreplicated DNA triggers the G_2 replication checkpoint delay of mitotic initiation (Elledge, 1996; Feilotter *et al.*, 1992). The effects of low and high concentrations of sperm in Pin1-depleted or mock-depleted extracts are presented in Figure 11. The higher DNA concentration causes a G_2 delay in mock-depleted extracts. However, this delay is greatly reduced in Pin1-depleted extracts. Thus, the difference in timing of mitotic entry observed between mock-depleted and Pin1-depleted extracts may reflect the inability of Pin1-depleted extracts to arrest in G_2 in response to unreplicated DNA. Notably, when the concentration of DNA is low, the transition out of mitosis into interphase occurs normally even without Pin1. In contrast, the Pin1-depleted extract supplemented with DNA to achieve a high concentration of chromatin fails to exit mitosis. It is possible that the M phase arrest occurring in these

extracts does so because of mitosis is initiated the presence of unreplicated DNA, and therefore M phase failsafe mechanisms are triggered to prevent segregation of damaged chromosomes.

To test the hypothesis that the operation of the replication checkpoint requires Pin1, replication was specifically suspended by addition of the DNA polymerase inhibitor, aphidicolin, to interphase extracts. In mock-depleted extracts, aphidicolin treatment postpones mitotic entry as expected (Figure 12). Depletion of Pin1 from extracts or addition of caffeine, a treatment that, by inhibiting ATM/ATR disables that component of G_2 checkpoint function (Dasso and Newport, 1990; Patel *et al.*, 1997; Schlegel and Pardee, 1986; Zhou *et al.*, 2000a), prevents the aphidicolin-induced cell cycle delay.

That Pin1 is directly responsible for the failure of the replication checkpoint delay of mitotic entry is indicated by the observation that supplementation of Pin1-depleted extracts with recombinant xPin1 restores the G_2 delay elicited by aphidicolin (Figure 13A, dashed line). This delay remained caffeine-sensitive in the reconstituted extract (data not shown), attesting to the restoration of a functional ATM/ATR-dependent checkpoint response. Furthermore, the catalytically inactive point mutant of Pin1, xPin1^{C109A}, does not complement the checkpoint defect (Figure 13A, asterisks), indicating that the prolyl isomerase activity of the enzyme is essential for replication checkpoint integrity in *Xenopus*.

It was important to determine whether the premature mitosis that occurs in the absence of Pin1 is indicative of more than a kinetic change in cell cycle progression. In all assays examined, mitosis observed in Pin1-depleted extract is indistinguishable from that seen in the presence of caffeine. Hyperphosphorylation of Cdc25, increased H1 kinase activity, and the appearance of MPM-2 epitopes accompanies microscopically observed mitotic entry (Figure 13B and Figure 10). Therefore, Pin1 is not required for MPM-2 epitope generation *per se*, or for the ability of mitotic phosphoproteins to regulate mitotic progression. Instead, precocious activation of Cdc25 may be the direct consequence of Pin1 removal.

It has been reported that Pin1 antagonizes *in vitro* phosphorylation of the mitotic regulators, Cdc25, Myt1 and Wee1 by Cdc2/cyclin B (Patra *et al.*, 1999). Although the ability of Pin1 to bind mitotic phosphoproteins appears to be important for this inhibition, association alone is not sufficient for endogenous Pin1 function. While the xPin1^{C109A} mutant binds to Pin1-binding proteins as well or better than the wild type protein (Figure 8A), this mutant is incapable of restoring the checkpoint response in Pin1-depleted extracts when added to achieve concentrations sufficient for complementation by wild type xPin1. Furthermore, *Xenopus laevis* Pin1 complements the lethality of *ess1* mutants in budding yeast, but the catalytically compromised mutant is inactive in the complementation assay[1]. Together these observations indicate that Pin1 is functionally conserved and that its catalytic activity is required for both its checkpoint role in *Xenopus* and its essential function in *S. cerevisiae*.

Target-specific inhibition of Cdc2 activity may offer a biochemical mechanism for Pin1's role in enabling the replication checkpoint. It has been suggested Cdc25 hyperphosphorylation by the Cdc2/cyclin B1 complex is directed by p13/SUC1's affinity for Cdc25's N-terminal phosphoepitopes (Landrieu *et al.*, 2001). The targeting of Cdc2/cyclin B1, through its stable association with p13/SUC1, to Cdc25 is competitively antagonized by Pin1 binding to the phosphatase and disrupting p13/SUC1-binding (Patra *et al.*, 1999), potentially explaining the conflicting data regarding whether Pin1 association inhibits Cdc25 activity (Crenshaw *et al.*, 1998; Shen *et al.*, 1998; Stukenberg and Kirschner, 2001; Zhou *et al.*, 2000b). Thus, Pin1-mediated inhibition of Cdc25 hyperphosphorylation likely acts in concert with 14-3-3-mediated cytoplasmic sequestration of the phosphatase, and perhaps

[1] C. B. Wilcox, K. E. Winkler, A. R. Means and S. D. Hanes, unpublished data.

with isomer-specific PP2A dephosphorylation of Cdc25's activating phosphorylations enabled by Pin1 (Zhou *et al.*, 2000b), to prolong G_2 by preventing functional interaction of Cdc25 with Cdc2 under checkpoint conditions. This function is consistent with previous observations that G_2 is temporally extended when Pin1 is over-expressed (Crenshaw *et al.*, 1998; Lu *et al.*, 1996; Shen *et al.*, 1998).

In the absence of DNA perturbation, consequences of Pin1-removal may not be manifested; this would explain the lack of gross cell cycle disruption in Pin1-null *Drosophila* and mouse mutants. Demonstration of the essential role of Pin1 in the caffeine-sensitive replication checkpoint establishes a position for endogenous Pin1 in the eukaryotic cell cycle regulatory network.G_2 checkpoints provide failsafe protection in normally dividing cells (Elledge, 1996), but they also influence the genomic instability common in cancers and may affect the efficacy of certain cancer therapies (Hartwell, 1992; Maity *et al.*, 1997; O'Connor, 1997; Piette and Munoz, 2000). Neoplastic transformation is characterized by autonomous cell division, and many commonly-used therapeutic agents target proliferative cells by capitalizing on their enhanced vulnerabilities to DNA damage. Typically, a cell with damaged DNA will afford itself time for repair prior to mitosis by activating the cdc25-dependent checkpoint in parallel with a second G_2 checkpoint that functions through the activity of p53. However, in the progression to oncogenic transformation cells frequently lose p53 function, rendering the caffeine-sensitive checkpoint pathway critical for any chance at recovery from genetic lesions (Toyoshima *et al.*, 1998). Of course, the goal of the oncologic therapies is to prevent this recovery, and to do so selectively. Disabling the cdc25-dependent G_2 checkpoint would, in theory, sensitize $p53^{-/-}$ cancer cells to DNA damage while sparing normally dividing cells that maintain functional p53 (Shapiro and Harper, 1999). Experiments in cultured cells demonstrate the potential efficacy of such a strategy (Blasina *et al.*, 1997; Suganuma *et al.*, 1999).

Pin1 may be an attractive target in this endeavor because we have shown that its catalytic activity is required for the Cdc25-dependent G_2 checkpoint. In accordance with this possibility is the observation that several approaches used to reduce Pin1 expression markedly induce apoptosis in transformed cell lines, while untransformed cells are more mildly affected (Rippmann *et al.*, 2000). Mechanistically, Pin1 seems to attenuate cdc25 activity by enhancing its dephosphorylation by PP2A, and it is likely that the premature mitotic entry observed in the absence of Pin1 is the manifestation of promiscuous activity of cdc25. Thus, one would expect that the loss of Pin1 function would be especially notable when cdc25 is overexpressed, as it often is in breast cancer. The increased need for Pin1 in such a scenario may select for the overexpression of Pin1, itself, in many cancers as has been documented here and elsewhere, further emphasizing the potential of this Pin1 as a target for adjuvant chemotherapeutics in cdc25-overexpressing cells.

Key Research Accomplishments

1. Cloning of the *X. laevis* homologue of Pin1
2. Prokaryotic expression of recombinant xPin1 and antibody generation
3. Demonstration of Pin1's ability to bind Cdc25 and structure-function analysis of the interaction
4. Evaluation of exogenous Pin1's influence on cell cycle progression in *X. laevis* extracts
5. Demonstration that Pin1 is not required for mitotic exit
6. Recognition that Pin1 modulates the timing of mitotic entry
7. Demonstration that Pin1 is required for the caffeine-sensitive replication checkpoint
8. Demonstration that this requirement relies on the enzymes prolyl isomerase activity
9. Recognition that premature mitosis in the absence of Pin1 is characterized by the appearance of hyperphosphorylated cdc25 and activate histone H1 kinase activity

Reportable Outcomes

1. **Thesis:** "*Pin*ning down the Cell Cycle: An Examination Of G_2/M Regulation By PIN1 And Its Binding Partners," by Katharine Estelle Winkler, Department of Pharmacology, Program in Cell and Molecular Biology, Duke University, Durham, North Carolina. Dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology in the Graduate School of Duke University, copyright 2001.
2. **Manuscript:** Katherine I. Swenson, Katharine E. Winkler, Anthony R. Means. Working title: MLK3: A NIMA-like cell cycle regulated kinase localized near the centrosome. *Submitted.*
3. **Manuscript:** Katharine E. Winkler, Katherine I. Swenson, Sally Kornbluth, Anthony R. Means. 2000. Requirement of the Prolyl Isomerase Pin1 for the Replication Checkpoint. *Science*, 287: 1644-1647. **See Appendix A.**
4. **Manuscript:** Jing Yang, Katharine Winkler, Minoru Yoshida, Sally Kornbluth. 1999. Maintenance of G_2 arrest in the *Xenopus* oocyte: a role for 14-3-3-mediated inhibition of Cdc25 nuclear import. *EMBO*, 18: 2174-2183. **See Appendix B.**
5. **Abstract and Poster:** Keystone Symposium: Cell Cycle 2001, January 2001.
6. **Abstract and Poster:** Era of Hope Department of Defense Breast Cancer Research Program Meeting, June 2000.
7. **Abstract and Poster:** Keystone Symposium: Cancer, Cell Cycle and Therapeutics, January 2000.
8. **Award Lecture:** Harold M. Weintraub Graduate Student Award Symposium "Pinning Down the Cell Cycle" Fred Hutchinson Cancer Research Center, Seattle, WA, May 2000.
9. **Invited Talk:** Biological Sciences Graduate Student Symposium "The Replication Checkpoint Requires the Prolyl Isomerase, Pin1" Duke University, Durham, NC, October 1999.
10. **Invited Talk:** Pharmacology and Cancer Biology Departmental Retreat "The Replication Checkpoint Requires the Prolyl Isomerase, Pin1" Duke University, Durham, NC, April 1999.

Conclusions

Globally, in combination with published data from other laboratories, these studies imply a role for isomerization in manifesting the effects of proline-directed phosphorylation that is a common modification among mitotic regulators. Structural modulation of proteins that results from phosphorylation is often invoked to explain the regulatory influence of this post-translational modification. Phosphate attachment to amino acid side chains can directly affect secondary, tertiary, and quaternary protein structure, as well as provide signature motifs recognized by protein binding domains. The existence of a phosphate-directed peptidyl-prolyl *cis-trans* isomerase opens the possibility that isomerization is another way in which phosphate causes changes in protein function. In some cases, a regulated phosphorylation event might exert its influence in cooperation with protein remodeling enzymes. The recognition that some proteins exhibit isomer-specific recognition of phosphorylated sequences supports the likelihood that phosphorylation-directed prolyl isomerization is a *bone fide* signaling mechanism.

In the context of short peptides, phosphorylation of serine/threonine-proline motifs has been shown to stabilize the *cis* conformation of the peptide (Schutkowski *et al.*, 1998). By limiting spontaneous isomerization, phosphorylation may not only render a serine/threonine-proline site a Pin1 substrate, but also heighten the need for Pin1's catalytic influence on backbone dynamics by rendering a proline resistant to immunophilin action (Yaffe *et al.*, 1997). Known consequences of Pin1-mediated isomerization include effects on target proteins' catalytic activity (Stukenberg and Kirschner, 2001; Wulf *et al.*, 2001), substrate preference (Patra *et al.*, 1999), associative interactions (Lu *et al.*, 1999a), phosphorylation state (Liu *et al.*, 2001; Zhou *et al.*, 2000b), acetylation (Arevalo-Rodriguez *et al.*, 2000), and proteolysis (Hsu *et al.*, 2001). Furthermore, the proline-directed p42 MAP kinase favors substrates with a *trans* prolyl bond adjacent to the phospho-acceptor (Weiwad *et al.*, 2000), and PP2A dephosphorylates *trans* isomers preferentially (Zhou *et al.*, 2000b). A role for phospho-serine/threonine-directed prolyl isomerization in manifesting conformational changes induced by phosphorylation could be widespread.

The restraining influence of phosphorylation on the conformation of prolyl peptide bonds within proteins has not been clearly demonstrated. However, indirect evidence that the conclusions from peptide assays accurately reflect protein biochemistry can be extracted from the experiments addressing Pin1's effects on Cdc25 conformation (Stukenberg and Kirschner, 2001). These researchers show that exposure of phosphorylated Cdc25 to sub-stoichiometric quantities of Pin1 not only directly affects the structure of the phosphatase as judged by limited protease digestion, but also is required for its recognition by the MPM-2 antibody. If isomerization by Pin1 is necessary to render a phospho-serine/threonine-proline motif an MPM-2 epitope, the monoclonal antibody, itself, might become a tool for identifying Pin1's mitotic targets. Furthermore, an MPM-2-directed phosphatase activity has been partially purified from *Xenopus* oocyte extracts (Che *et al.*, 1998). The activity was not attributed to a known phosphatase, and future studies may identify this as a dedicated MPM-2 phosphatase. A conformation-dependent MPM-2 phosphatase would be an attractive candidate to investigate in unraveling the mechanism by which Pin1 negatively influences mitotic entry.

Currently, it is clear that Pin1 has in an explicit G_2/M regulatory role that requires its activity as a peptidyl-prolyl *cis-trans* isomerase. The experiments described here are a starting point for what promises to be an exciting investigation of cell cycle regulation by the mitotic kinases. Broader implications may also present themselves as inquiries of these sorts lead to an appreciation of the potential for proline isomerization in biological decision-making.

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A

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          10          20          30          40          50          60
ATG GCG GAC GAG GAG AAG CTA CCG CCC GGG TGG GAG AAG CGA ATG AGC CGA AGC TCT GGG
 M   A   D   E   E   K   L   P   P   G   W   E   K   R   M   S   R   S   S   G>

          70          80          90          100         110         120
CGT GTA TAC TAT TTC AAT CAC ATG ACC AAC GCC AGC CAA TGG GAA AGG CCC ATC GCC GGT
 R   V   Y   Y   F   N   H   M   T   N   A   S   Q   W   E   R   P   I   A   G>

          130         140         150         160         170         180
GGG AAG AAT GGC CAA GGG GAG CCG GGT AAA GTG CGG TGT TCT CAT CTA CTT GTG AAG CAC
 G   K   N   G   Q   G   E   P   G   K   V   R   C   S   H   L   L   V   K   H>

          190         200         210         220         230         240
AAT CAG TCA CGG CGC CCT TCA TCA TGG AGG CAG GAC AAG ATC ACA CGC AGC AAG GAC GAG
 N   Q   S   R   R   P   S   S   W   R   Q   D   K   I   T   R   S   K   D   E>

          250         260         270         280         290         300
GCG CTG GAA CTC ATT AAT GGC TAC ATC CAG AAG ATG AAA TCG GGG GAT GAG GAT TTC GAA
 A   L   E   L   I   N   G   Y   I   Q   K   M   K   S   G   D   E   D   F   E>

          310         320         330         340         350         360
TCT CTT GCT TCT CAG TTC AGT GAC TGC AGC TCA GCT AAG GCC GGA GGG GAT CTG GGC AGC
 S   L   A   S   Q   F   S   D   C   S   S   A   K   A   G   G   D   L   G   S>

          370         380         390         400         410         420
TTT GGG AAA GGT GCA ATG CAG AAG CCG TTT GAA GAT GCT TCT TTC GCT CTG AGG CCT GGA
 F   G   K   G   A   M   Q   K   P   F   E   D   A   S   F   A   L   R   P   G>

          430         440         450         460         470         480
GAG ATG AGT GGC CCC GTC TTC ACA GAT TCC GGC ATT CAC ATT ATC CTC CGC ACT GAG TGA
 E   M   S   G   P   V   F   T   D   S   G   I   H   I   I   L   R   T   E   *>
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B

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Xl Pin1     1   MADEEKLPPGWEKRMSRSSGRVYYFNHMTNASQWER  36
Hs Pin1     1   MADEEKLPPGWEKRMSRSSGRVYYFNHITNASQWER  36
Dm Dodo     1   MPDAEQLPDGWEKRTSRSTGMSYYLNMYTKESQWDQ  36
An PinA     1   MVN-PGLPAGWEVRHSNSKNLPYYFNPATRESRWEP  35
Sc Ess1     1 MPSDVASRTGLPTPWTVRYSKSKKREYFFNPETKHSQWEE  40

Xl Pin1    37 P-----------------IAGGKNGQG---EPGKVRCSHLLV  58
Hs Pin1    37 PSG-----------NSSSGGKNGQG---EPARVRCSHLLV  62
Dm Dodo    37 PTEP----------AKKAGGGSAGGGD--APDEVHCLHLLV  65
An PinA    36 PADTDMETLKMYMATYHSGAATYHEAPSQEGKIRCSHLLV  75
Sc Ess1    41 PEG-------------TNKDQLHKHLRDHPVRVRCLHILI  67

Xl Pin1    59 KHNQSRRPSSWRQDKITRSKDEALELINGYIQKMK--SGD  96
Hs Pin1    63 KHSQSRRPSSWRQEKITRTKEEALELINGYIQKIK--SGE 100
Dm Dodo    66 KHKGSRRPSSWREANITRTKEEAQLLLEVYRNKIV--QQE 103
An PinA    76 KHRDSRRPSSWREAEITRTKEEARELRGHQERIM--RGE 113
Sc Ess1    68 KHKDSRRPA'SHRSENITISKQDATDELKTLITRLDDDSKT 107

Xl Pin1    97 EDFESLASQFSDCSSAKAGGDLGSFGKGAMQKPFEDASFA 136
Hs Pin1   101 EDFESLASQFSDCSSAKARGDLGAFSRGQMQKPFEDASFA 140
Dm Dodo   104 ATFDELARSYSDCSSAKRGGDLGKFGRGQMQAAFEDAAFK 143
An PinA   114 IRLGDLAMSESDCSSARKKGDLGFFGRGEMQKEFEEAAFA 153
Sc Ess1   108 NSFEALAKERSDCSSYKRGGDLGWFGRGEMQPSFEDAAFQ 147

Xl Pin1   137 LRPGEMSGPVFTDSGIHIILRTE 159
Hs Pin1   141 LRTGEMSGPVFTDSGIHIILRTE 163
Dm Dodo   144 LNVNQLSGIVDSDSGLHIILRKA 166
An PinA   154 LQPGQVSDIVESGSGLHLIERLQ 176
Sc Ess1   148 LKVGEVSDIVESGSGVHVIKRVG 170
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Figure 1. Sequence of the *Xenopus laevis* Pin1 homologue.
(A) cDNA sequence obtained in three independent clones identified by low stringency hybridization using human Pin1 probe. (B) Amino acid sequence alignment of Pin1 homologues; identical and similar residues are shaded (dark and light, respectively).

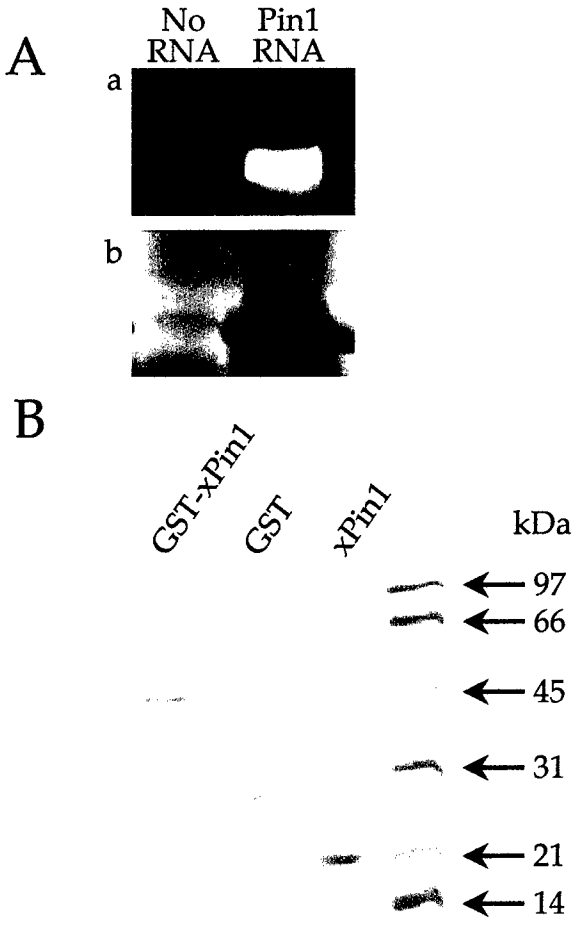

Figure 2. cDNA cloning and expression of xPin1.
(A) Ethidium bromide-stained RNA resolved in TAE/1% agarose (a) and ^{35}S-methionine-labeled protein separated by SDS-PAGE produced pBKCMV-xPin1$^{clone\ 6}$ fromT3 polymerase-driven *in vitro* transcription (a) and subsequent *in vitro* translation (b). (B) Coomassie blue staining of 1 μg recombinant GST-xPin1 and cleaved xPin1 purified from bacteria.

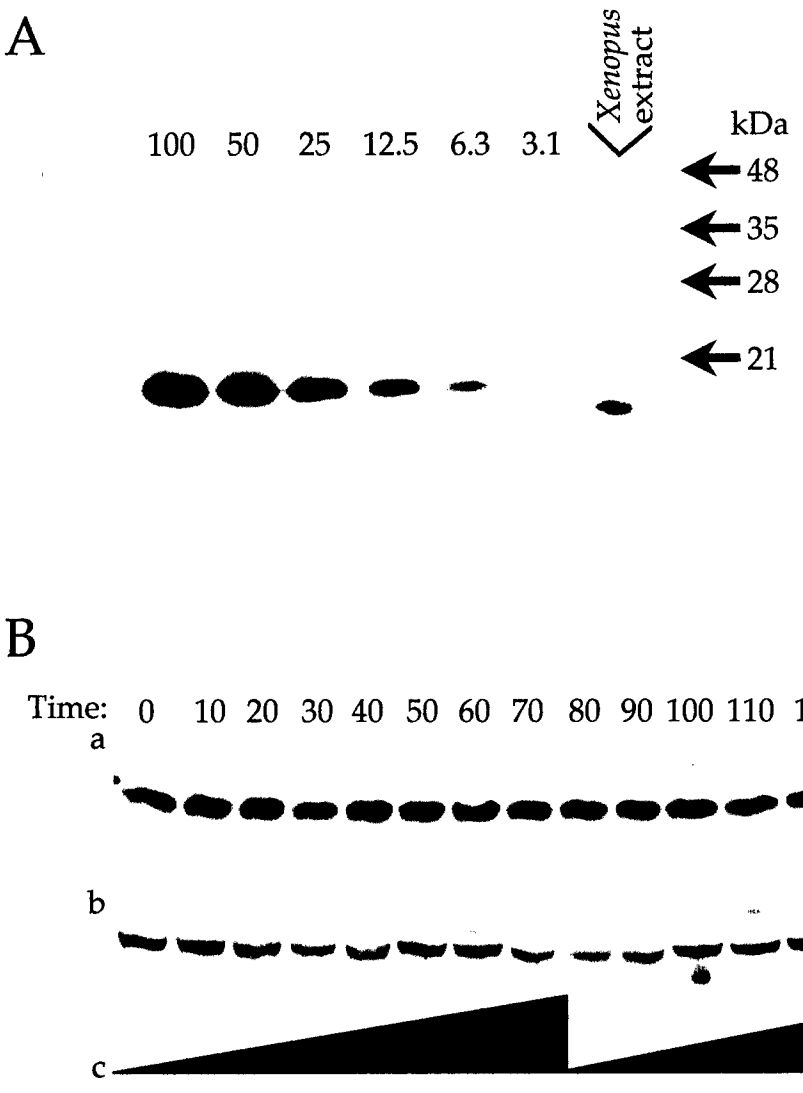

Figure 3. Pin1 expression in *Xenopus* extracts.
(A) Immunoblot using anti-xPin1 serum (diluted 5000-fold) of indicated quantities (ng) purified recombinant xPin1 and 0.5 μL *Xenopus* egg extract. (B) Immunoblots of Pin1 (a) and cdc25 (b) expression and representation of histone H1 kinase activity (c) in cycling *Xenopus* extract.

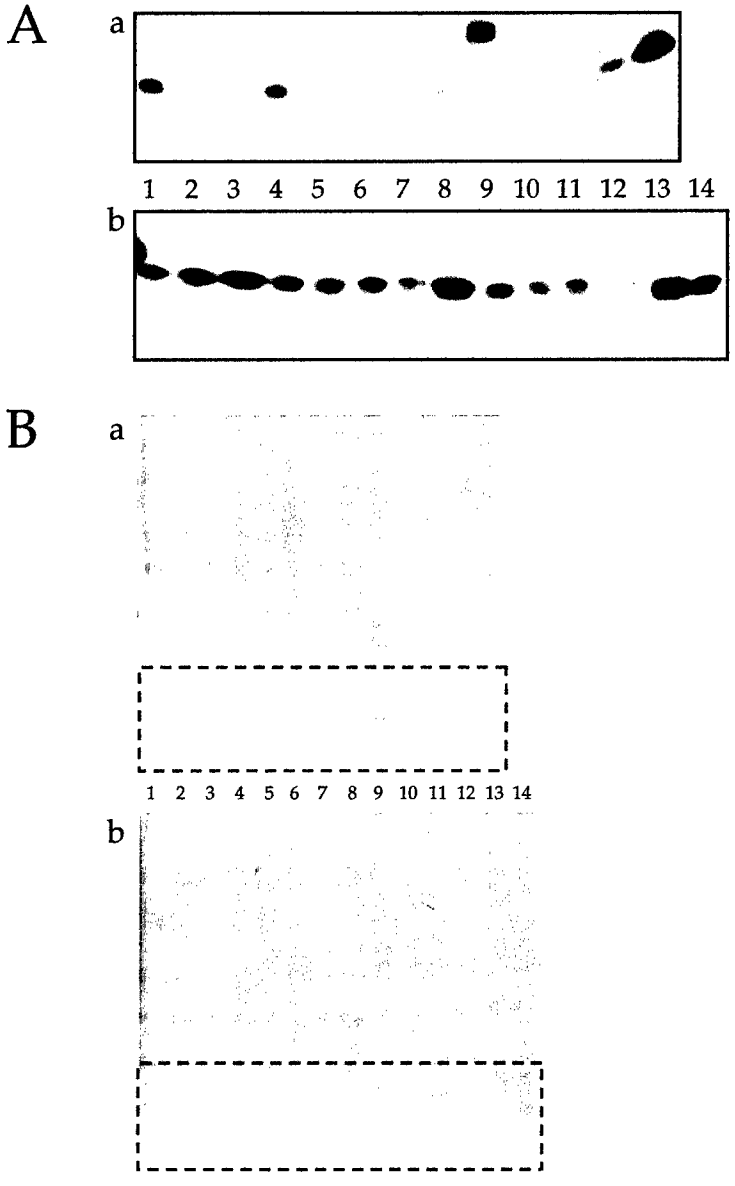

Figure 4. Pin1 expression within and among mammalian tissues.
(A) Pin1 immunoblot analysis of 75 μg soluble protein extracts generated from adult (a) and neonatal (b) mouse tissue: cerebrum (1); cerebellum (2); heart (3); kidney (4); liver (5); large intestine (6); lung (7); muscle (8); pancreas (9); small intestine (10); spleen (11); stomach (12); testis (13); thymus (14). (B) Membranes stained with coomassie blue confirm equal loading except where degradation is apparent in (a)10 and, to lesser extent, in (b)7 and (b)12. Regions exposed for (A) are indicated.

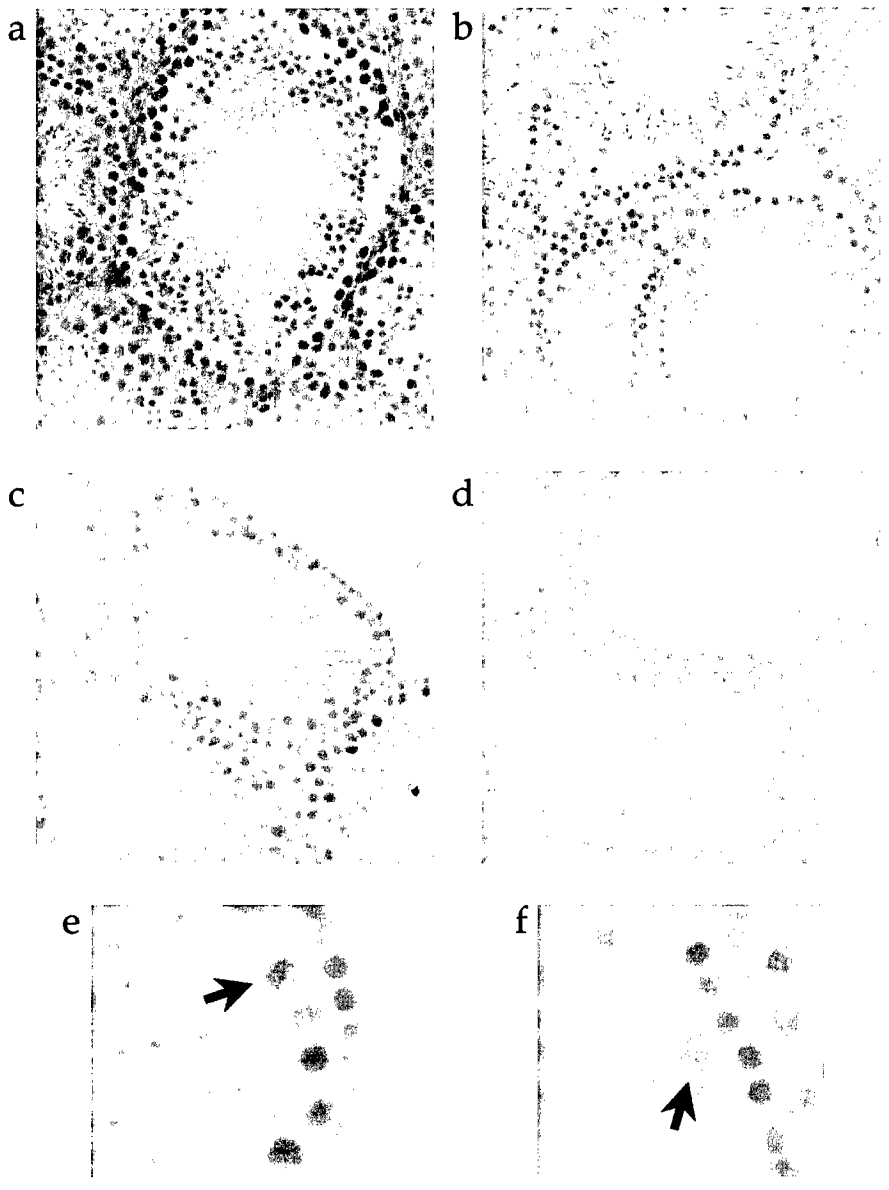

Figure 5. Pin1 staining of testis from wild type (a, c, and e) and $Pin1^{-/-}$ (b, d, and f) mice. Brown stain, Pin1; green/blue stain, DNA; pachytene spermatocytes, arrows.

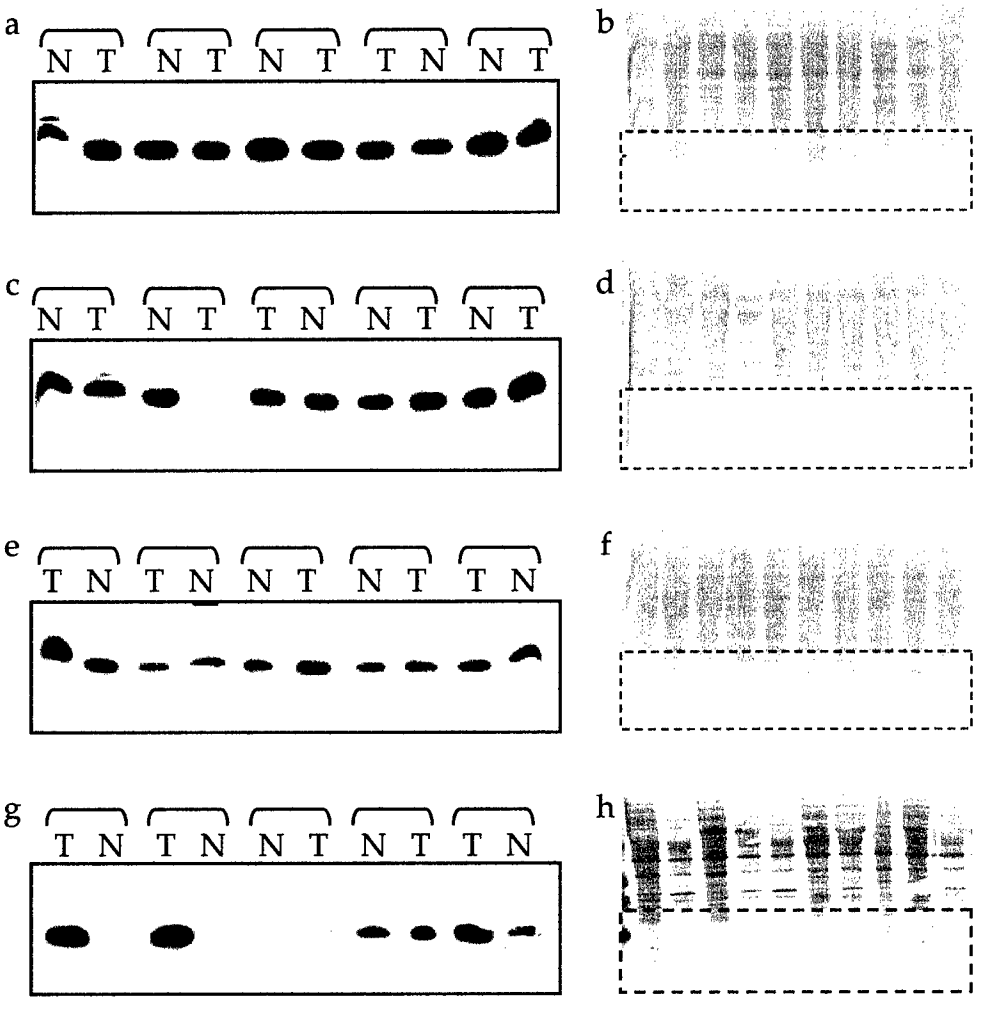

Figure 6. Expression of Pin1 in primary human tumors.
Soluble extracts (75 μg) of tumor (T) or adjacent normal (N) tissue samples from five human colon (a), kidney (c), liver (e), and lung (g) cancers were immunoblotted for Pin1. Pairs of samples were coded for unbiased analysis. Membranes stained with coomassie blue are shown to the right of the appropriate immunoblot (b, d, f, and h), with the exposure area indicated.

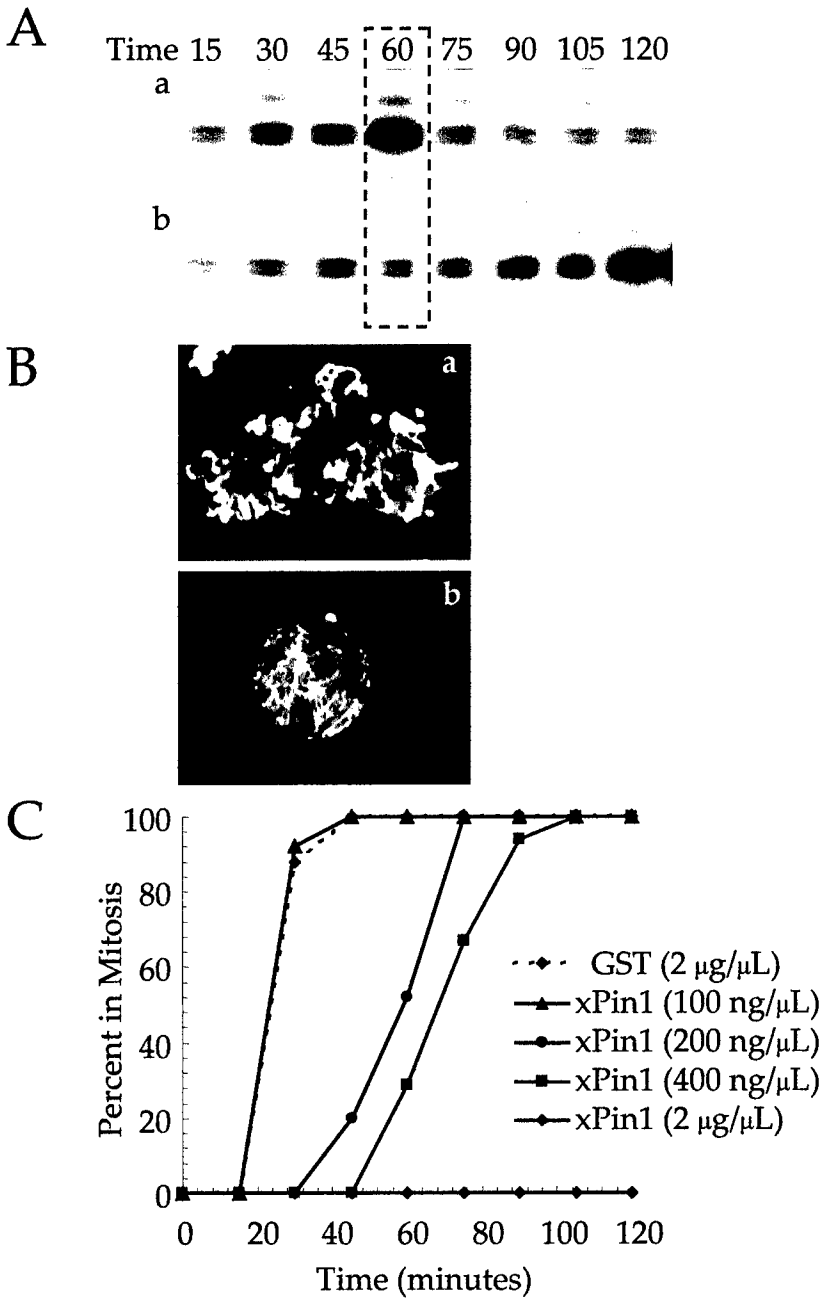

Figure 7. Mitotic delay upon addition of recombinant xPin1 to *Xenopus* extracts. (A) Autoradiogram of phosphate incorporation into histone H1 and (B) representative DNA staining in aliquots removed from cycling extracts at the indicated time (min) following extract supplementation with demembranated sperm chromatin, ATP regenerating mix, and GST (a) or xPin1 (b) protein and transfer to 25°C. (C) Mitotic index in S extract supplemented with chromatin, ATP regenerating mix, non-degradable human cyclin B1, and GSTor xPin1 to final concentrations indicated ranging form 5-fold to 100-fold over endogenous levels of 20 ng/μL).

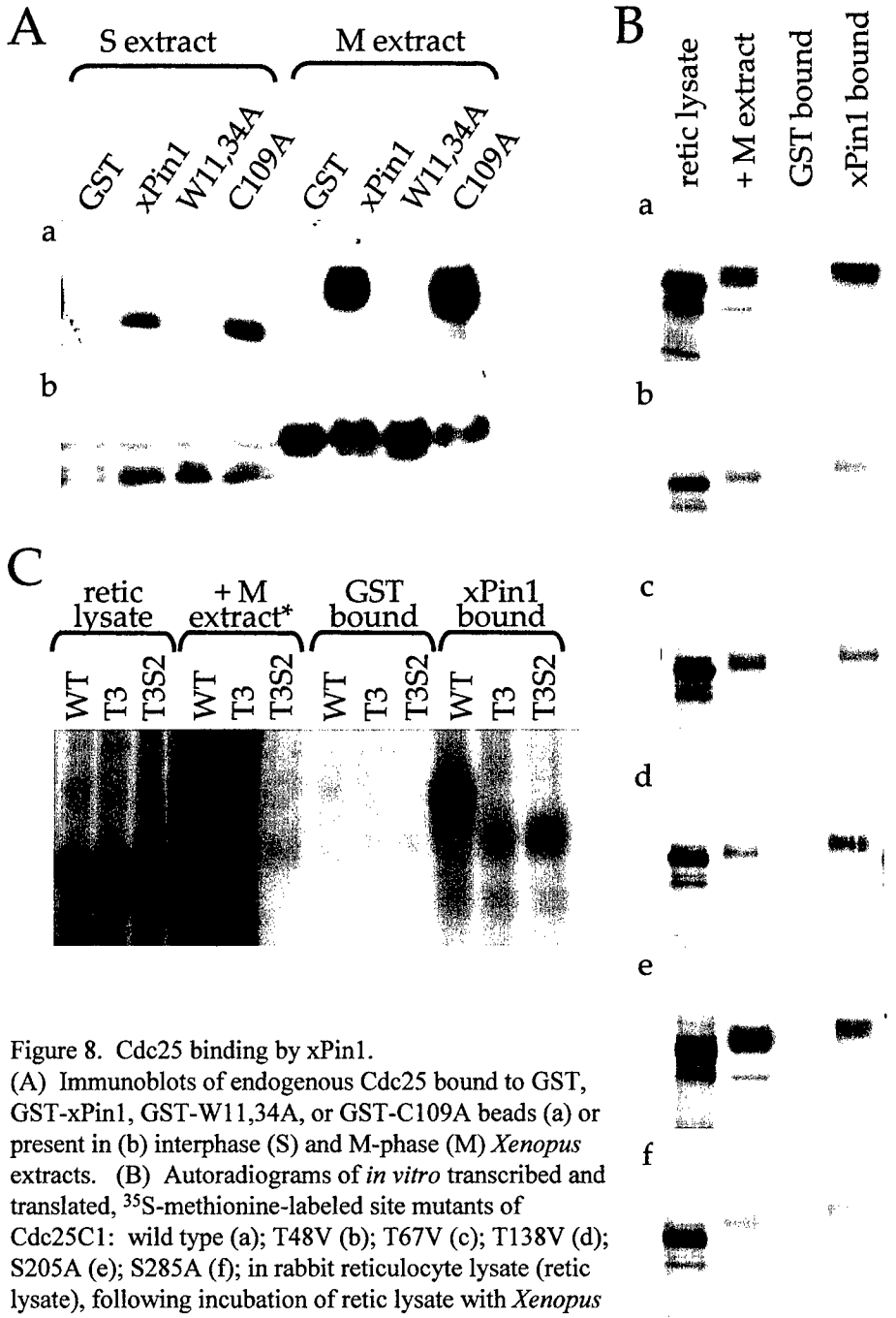

Figure 8. Cdc25 binding by xPin1.
(A) Immunoblots of endogenous Cdc25 bound to GST, GST-xPin1, GST-W11,34A, or GST-C109A beads (a) or present in (b) interphase (S) and M-phase (M) *Xenopus* extracts. (B) Autoradiograms of *in vitro* transcribed and translated, ^{35}S-methionine-labeled site mutants of Cdc25C1: wild type (a); T48V (b); T67V (c); T138V (d); S205A (e); S285A (f); in rabbit reticulocyte lysate (retic lysate), following incubation of retic lysate with *Xenopus* M extract cytosol in the presence of ATP for 20 min at 25°C (+ M extract), or the material bound to GST or GST-xPin1 beads from twice the amount of retic loaded.
(C) Autoradiogram of similar experiment performed for multiple site mutants of Cdc25C1: wild type (WT); T48,67,138V (T3); T48,67,138V;S205,285A (T3S2).
* High protein concentrations in these lanes caused distortion.

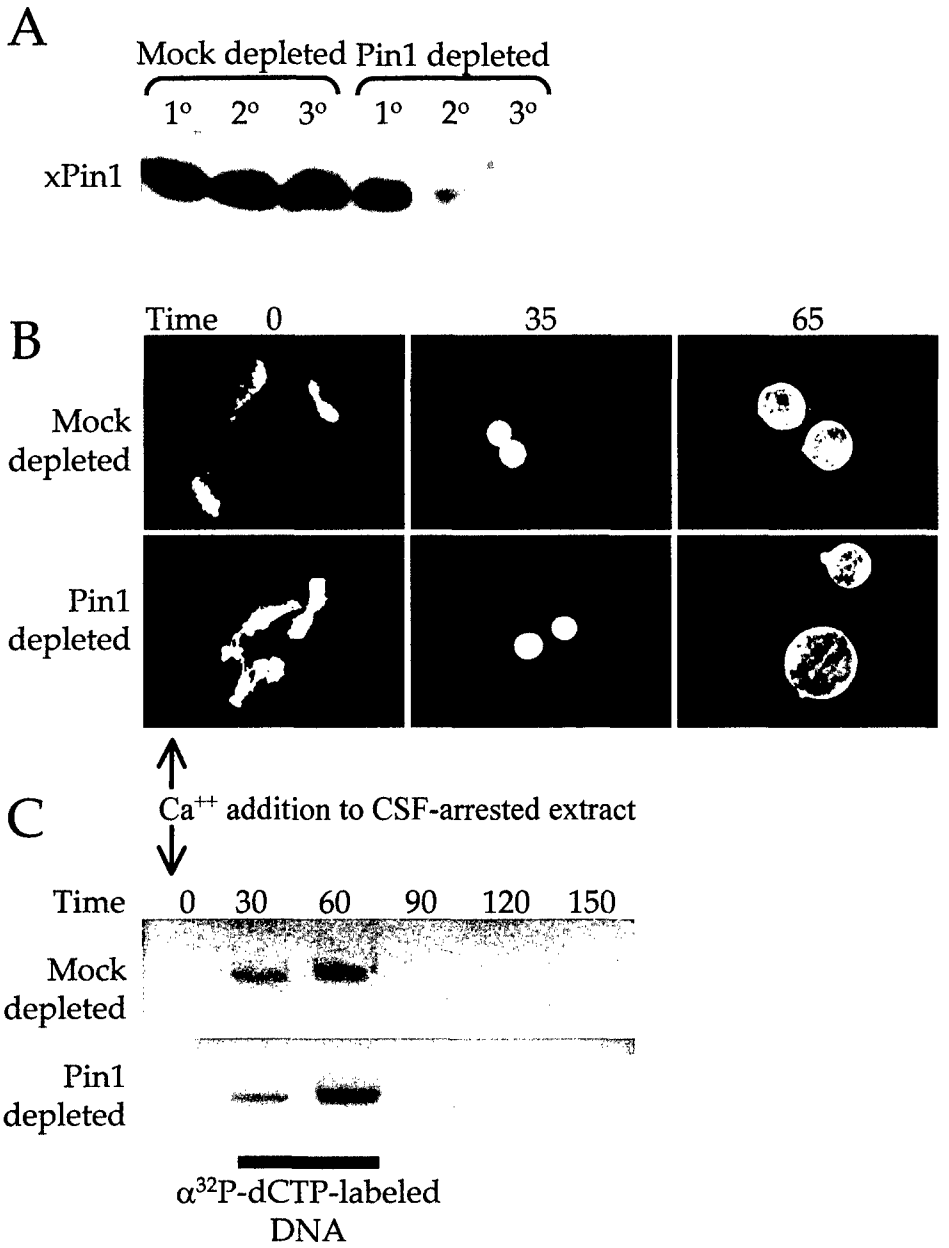

Figure 9. Exit from mitosis in *Xenopus* extracts lacking Pin1.
(A) Immunoblot of Pin1 in CSF extracts immunodepleted three times in succession (1°, 2°, 3°). (B) DNA visualized at indicated times (min) following supplementation of 3°depleted extracts with demembranated sperm chromatin, and ATP regenerating mix, and 400 μM $CaCl_2$. (C) Autoradiogram of α^{32}P-dCTP incorporated into DNA by pulse labeling in extracts and resolved by agarose gel electrophoresis.

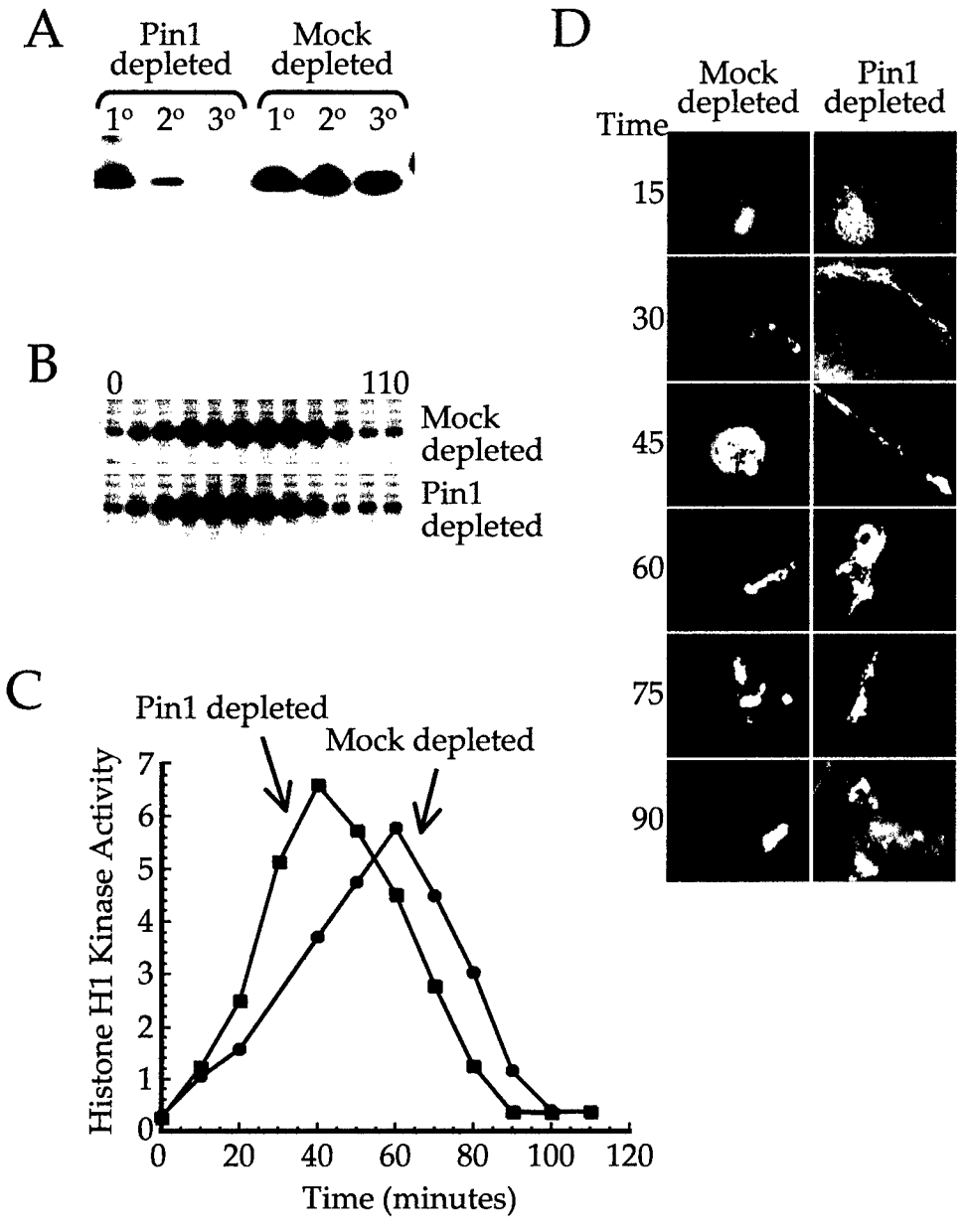

Figure 10. Function for Pin1 in the regulation of the G_2/M transition.
(A) Immunoblot confirming depletion of Pin1 from S extracts. (B) Autoradiogram and (C) Phosphorimager quantification of histone H1 kinase activity in aliquots of 3°-depleted extracts at indicated times (min) after supplementation with demembranated sperm nuclei, ATP, and Δcyclin B1. (D) Typical images of nuclei from a separate but similar experiment.

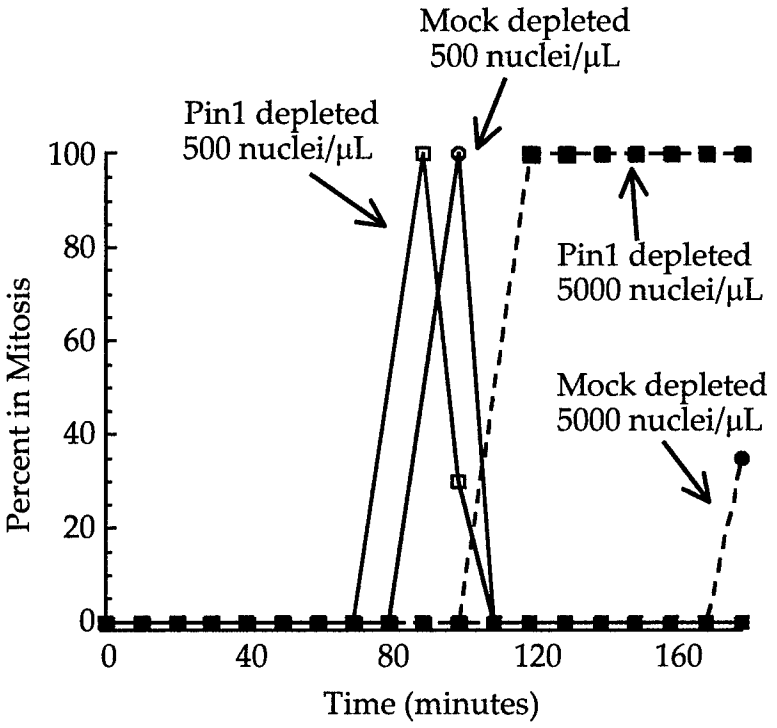

Figure 11. Potentiation of the Pin1 depletion effect by addition of extra chromatin. Mitotic index, monitored by fluorescence microscopy, in cycling extracts depleted of Pin1 and supplemented with ATP and 500 or 5000 nuclei/μL..

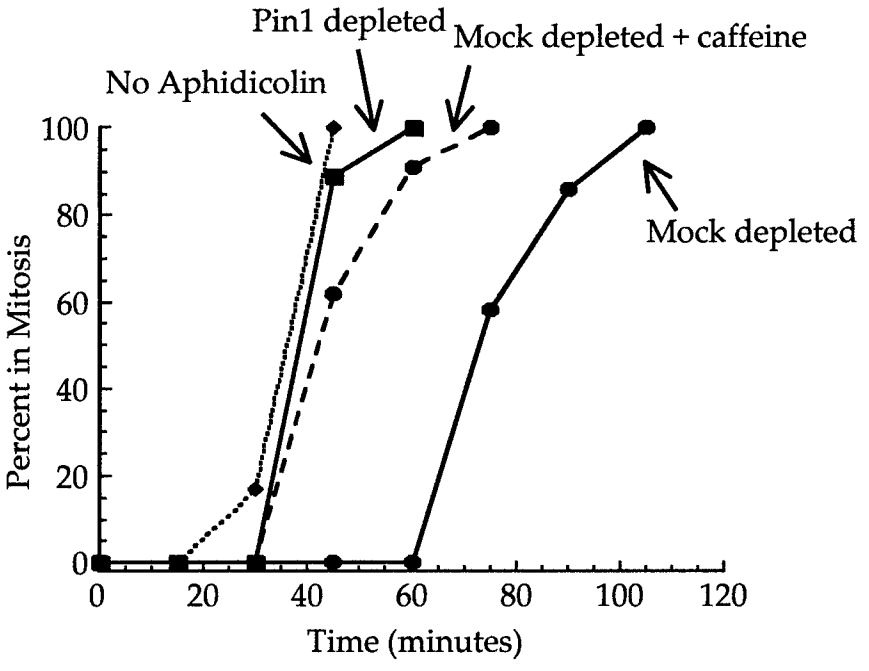

Figure 12. Requirement of Pin1 for the checkpoint arrest in response to unreplicated DNA. Mitotic index at specified time after Δcyclin B1 addition to immunodepleted cytosolic S extract supplemented with membranes isolated from interphase egg extracts (1:10), demembranated sperm nuclei, ATP regenerating mix, and aphidicolin (50 μg/mL, except as indicated, dotted line), in the presence (dashed line) or absence (solid lines) of caffeine (5 mM).

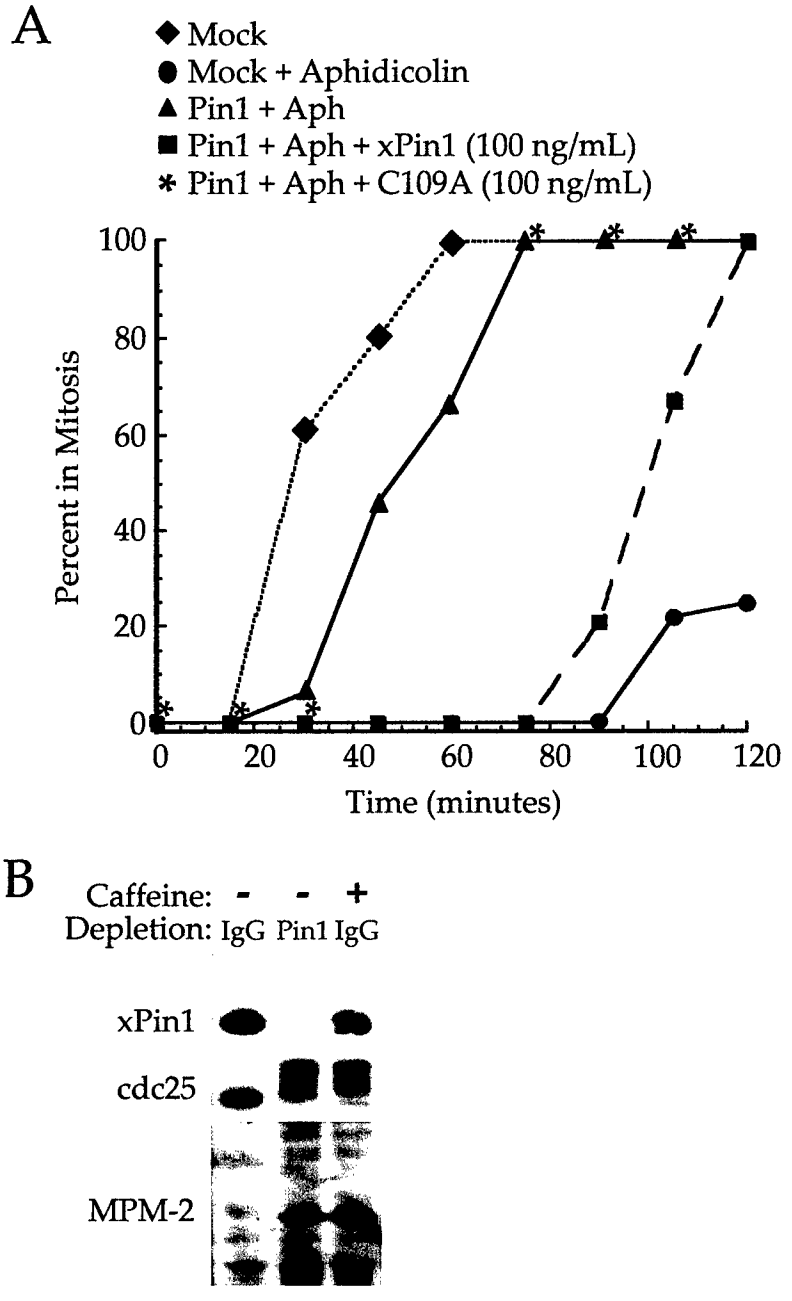

Figure 13. Restoration of checkpoint function after addition of recombinant xPin1. (A) Mitotic index of extracts treated as in Figure 4.4, and supplemented with xPin1 or C109A protein (100 ng/μL). (B) Immunoblots showing xPin1, cdc25, and MPM-2 reactivity in the above extracts following 120 min in the presence of Δcyclin B1.

Appendix A

[K. Kusano, R. Miledi, J. Stinnakre, *J. Physiol (London)* **328**, 143 (1982)] and 101 mM (based on the fitting of the Nernst plot in Fig. 2E), respectively.
13. However, the possibility exists that Kcv functions as a subunit, which up-regulates the activity of endogenous K^+ channels in oocytes. To exclude this possibility, we constructed a site-specific mutation in the selectivity filter sequence of Kcv by replacing Phe^{66} (F66) with Ala (A). If Kcv is a channel protein, the mutant protein should, by analogy to the Shaker channel, form a channel unable to conduct K^+ currents (*1*). Using standard voltage-clamp assays, nine oocytes expressing KcvF66A had currents similar in kinetics to those of H_2O-injected oocytes with no additional K^+ conductance compared to the H_2O-injected control cells. Hence, the absence of a prominent K^+-selective current in KcvF66A-expressing oocytes confirms that Kcv functions as a channel protein in oocytes and that the observed currents are not due to activation of endogenous channel proteins.
14. G. Yellen, *Curr. Opin. Neurobiol.* **9**, 267 (1999); D. M. Papazian, *Neuron* **23**, 7 (1999).
15. C. Wang, K. Takeuchi, L. H. Pinto, R. A. Lamb, *J. Virol.* **67**, 5585 (1993).
16. Concentration for half-maximal inhibition estimated by fitting Michaelis-Menten type kinetics to data.
17. The adamantyl group of amantadine is believed to interact with the hydrophobic lining of the M2 pore, whereas the ammonium group forms H bonds with the imidazole ring of His^{37} [C. S. Gandhi *et al.*, *J. Biol. Chem.* **274**, 5474 (1999)].
18. Voltage-dependency of inhibition by Ba^{2+} was analyzed quantitatively based on a Woodhull block model [A. M. Woodhull, *J. Gen. Physiol.* **61**, 687 (1973)] by fitting data of relative block to

$$\left(1 - \frac{I}{I_o}\right) = \frac{b_{max}}{1 + \frac{k^0}{B_a} e^{(z\delta F/RT)}}$$

where I_0 is control and I blocked current, b_{max} the maximal block, B_a the concentration of Ba^{2+}, k^0 the dissociation constant of the blocking reaction at voltage = 0 mV, δ the fraction of the electrical field crossed by Ba^{2+}, and $z = 2$ the valence of the blocking ion. R, T, and F have their usual thermodynamic meaning. Fitting yields for 1 mM Ba^{2+}: $b_{max} = 0.94$, $\delta = 0.9 \pm 0.03$, and $k^0 = 660 \pm 12$ μM ($n = 3$ oocytes).
19. R. Z. Sabirov, T. Tominaga, A. Miwa, Y. Okada, S. Oiki, *J. Gen. Physiol.* **110**, 665 (1997); R. C. Shieh, J. C. Chang, J. Arreola, *Biophys. J.* **75**, 2313 (1998); J. M. Owen, C. C. Quinn, R. Leach, J. B. Findlay, M. R. Boyett, *Exp. Physiol.* **84**, 471 (1999).
20. J. L. Van Etten, D. E. Burbank, Y. Xia, R. H. Meints, *Virology* **126**, 117 (1983).
21. About 1200 plaque-forming units of virus PBCV-1, or a small plaque variant P1210 [D. Landstein, D. E. Burbank, J. W. Nietfeldt, J. L. Van Etten, *Virology* **214**, 413 (1995)], were mixed with 10^8 host cells (*Chlorella* strain NC64A) and warm MBBM top agar containing various concentrations of ion-channel inhibitors. The mixture was layered onto MBBM nutrient agar (*22*). After incubating for 2 days at 25°C, viral plaques were counted. Potassium concentration of MBBM growth medium was 1.3 mM.
22. J. L. Van Etten, unpublished results.
23. R. A. Lamb and L. H. Pinto, *Virology* **229**, 1 (1997).
24. J. Kyte and R. F. Doolittle, *J. Mol. Biol.* **157**, 105 (1982).
25. Channel nomenclature according to (*7*) and A. Wei, T. Jegla, L. Salkoff, *Neuropharmacology* **35**, 805 (1996).
26. J. D. Thompson, D. G. Higgins, T. J. Gibson, *Nucleic Acids Res.* **22**, 4673 (1994); N. Saitou and M. Nei, *Mol. Biol. Evol.* **4**, 406 (1987). Only the pore region and flanking transmembrane segments were used for the phylogenetic comparisons. For Kir and Kv channels, consensus sequences of the appropriate subfamily were used in the calculations.
27. We thank H. Terlau, D. Gradmann, and M. Blatt for helpful discussions. Supported in part by MURST in the framework of Cofin-99, NIH grant GM32441 to J.V.E., Small Business Innovative Research grant GM41333 to M.N., Telethon grants (#971 and #296.bi) to D.D. and A.M., and a traveling grant by the SmithKline Beecham foundation to B.P.

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REPORTS

Requirement of the Prolyl Isomerase Pin1 for the Replication Checkpoint

Katharine E. Winkler, Katherine I. Swenson, Sally Kornbluth, Anthony R. Means*

The peptidyl-prolyl isomerase Pin1 has been implicated in regulating cell cycle progression. Pin1 was found to be required for the DNA replication checkpoint in *Xenopus laevis*. Egg extracts depleted of Pin1 inappropriately transited from the G_2 to the M phase of the cell cycle in the presence of the DNA replication inhibitor aphidicolin. This defect in replication checkpoint function was reversed after the addition of recombinant wild-type Pin1, but not an isomerase-inactive mutant, to the depleted extract. Premature mitotic entry in the absence of Pin1 was accompanied by hyperphosphorylation of Cdc25, activation of Cdc2/cyclin B, and generation of epitopes recognized by the mitotic phosphoprotein antibody, MPM-2. Therefore, Pin1 appears to be required for the checkpoint delaying the onset of mitosis in response to incomplete replication.

The peptidyl-prolyl isomerase (PPIase) Pin1 affects cell cycle transitions. Originally identified in yeast–two hybrid screens as a protein that binds to and suppresses the toxicity of the fungal mitotic kinase Never In Mitosis A (NIMA), Pin1 is present in all eukaryotic cells examined (*1–4*). Although Pin1 is an abundant protein, the expression of which does not change during the cell cycle (Fig. 1), it clearly influences cell cycle dynamics. Overexpression of Pin1 is deleterious in the budding yeast *Saccharomyces cerevisiae* and causes a G_2 arrest in HeLa cells and in *Xenopus laevis* egg extracts, suggesting that the protein negatively regulates the initiation of mitosis (*1, 3*). The budding yeast Pin1 homolog ESS1 is encoded by an essential gene; *ess1* deletion mutants exhibit terminal mitotic arrest, suggesting a requirement for Pin1 in mitotic exit (*4, 5*). In contrast, Pin1 is not critical for any readily observable function in *Drosophila melanogaster* (*2*) or mouse (*6*).

In vitro, Pin1 binds a subset of mitotic proteins containing a motif composed of a phosphoserine or phosphothreonine residue followed by a proline residue (*3, 7–9*) that is also recognized by the MPM-2 monoclonal antibody (*10, 11*). Among these potential cell cycle targets, only substoichiometric interaction of Pin1 with the mitotic phosphatase Cdc25C has been demonstrated in vivo (*12*); thus, it is unclear whether the numerous phosphoproteins associated with Pin1 in vitro are biologically relevant targets for Pin1 in vivo. Endogenous Pin1 protein has been implicated in transcriptional regulation and RNA processing in yeast (*5, 13–16*) and in mediating the association of phosphorylated tau with microtubules in brain extracts (*17*). The relation of these functions to control of the cell cycle remains unclear, and events regulated by Pin1 that influence the cell cycle have yet to be defined. We examined Pin1 function in *Xenopus* egg extracts that are transcriptionally inactive, thus allowing us to avoid possible effects of Pin1 on transcriptional events that might affect cell cycle progression. This model system provided the opportunity to focus on specific cell cycle transitions and thereby evaluate the contribution of Pin1 protein to each transition.

The *Xenopus* Pin1 homolog was isolated by low-stringency hybridization screening of a *Xenopus* gastrula cDNA library with a human Pin1 probe (*18*). The inserts of three independently isolated clones each encoded an identical open reading frame (*xPin1*). The predicted polypeptide sequence shared 89% identity with human Pin1 and >45% identity with each of the eukaryotic parvulins over its full length of 159 residues. Recombinant xPin1 was purified from bacteria (Fig. 1A) and used to generate polyclonal antiserum that recognized a single protein of 18 kD in *Xenopus* egg extracts (Fig. 1B). The concentration of Pin1 in egg extracts was estimated to be 20 ng/μl, or ~1 μM, and this did not change throughout the cell cycle (Fig. 1C).

The mitotic arrest observed in yeast lacking ESS1 suggested a function for the protein in mitotic exit. To test this directly, we used cytostatic factor–arrested egg extracts (CSF extracts) (*19*) to examine the consequences of the removal of Pin1 on mitotic exit and DNA replication. CSF extracts, generated in the presence of EGTA to prevent calcium-dependent degradation of cyclin B, exhibit high H1 kinase activity and other hallmarks of normal M phase arrest. Calcium addition, which recapitulates a physiological consequence of fertilization, causes the extracts to proceed into interphase, characterized by nuclear envelope formation, chromatin

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decondensation, and initiation of replication. When CSF extracts were immunodepleted of Pin1 (*20*), we found that Pin1-depleted and mock-depleted extracts were equally capable of exiting the M phase (Fig. 2, A and B). Furthermore, Pin1 depletion had no effect on DNA replication (Fig. 2C). Thus, in this cell-free system, Pin1 appears not to be required for either S phase or the M to G_1 transition.

Mitotic arrest observed in *ess1* mutant yeast could be the consequence of a premature mitotic entry triggered in the absence of Pin1. To examine the effects of Pin1 depletion on the isolated G_2 to M transition, we used both interphase extracts that were induced to enter M phase by the addition of exogenous cyclin B protein, and cycling extracts, which intrinsically oscillate between S and M phases of the cell cycle (*21*). Microscopic examination of nuclei, coupled with the measurement of Cdc2-cyclin B1–catalyzed histone H1 phosphorylation, revealed that both types of extract, when depleted of Pin1, entered mitosis more rapidly than did control extracts (Fig. 3, A through D). Although the absolute timing of mitotic entry varied from extract to extract, removal of Pin1 consistently accelerated the transition into M phase.

We explored the possibility that the premature mitosis in Pin1-depleted extract was due to the failure of a negative regulatory influence at the G_2 to M transition. The duration of interphase in *Xenopus* extracts can be prolonged by supplementing extracts with high concentrations of sperm chromatin, which increases the time required for DNA synthesis (*22*). The presence of unreplicated sperm DNA triggers the G_2 replication checkpoint that delays mitotic initiation by preventing activation of Cdc2 (*22–24*). The effects of low and high concentrations of sperm in Pin1-depleted or mock-depleted extracts were compared (Fig. 3D). The higher DNA concentration caused a G_2 delay in mock-depleted extracts. However, this delay was greatly reduced in Pin1-depleted extracts. Thus, the difference in timing of mitotic entry observed between mock-depleted and Pin1-depleted extracts may reflect the inability of Pin1-depleted extracts to halt mitotic entry in the presence of unreplicated DNA. Notably, when the concentration of DNA was low, the transition out of mitosis into interphase occurred normally, even without Pin1. In contrast, the Pin1-depleted extract that was supplemented with DNA to achieve a high concentration of chromatin failed to exit mitosis. It is possible that the M phase arrest in these extracts occurs because mitosis is initiated in the presence of unreplicated DNA, and therefore, M phase fail-safe mechanisms are triggered to prevent segregation of damaged chromosomes.

To test the hypothesis that the operation of the replication checkpoint requires Pin1, we suspended replication with the DNA polymerase inhibitor aphidicolin. In mock-depleted extracts, aphidicolin treatment postponed mitotic entry as expected (Fig. 4A). Depletion of Pin1 from extracts or addition of caffeine [a treatment that disables the replication checkpoint (*22, 25–27*)] prevented the aphidicolin-induced cell cycle delay. Supplementation of Pin1-depleted extracts with recombinant xPin1 restored the G_2 delay elicited by aphidicolin (Fig. 4B), and the delay remained caffeine sensitive in the reconstituted extract (*28*). This indicated that Pin1 itself is an essential component of the replication checkpoint in *Xenopus*.

Pin1's effects on cell cycle kinetics might be mediated through its established association with mitotic phosphoproteins (*3, 12*). For this reason, we examined the status of several Pin1-binding proteins in extracts depleted of Pin1. In all of our assays, the premature mitosis observed in Pin1-depleted extract was indistinguishable from that occurring in the presence of caffeine. Hyperphosphorylation of Cdc25, increased H1 kinase activity, and the appearance of MPM-2 epitopes (Figs. 3A and 4C) accompanied microscopically observed mitotic entry in both cases. Therefore, Pin1 is not required for MPM-2 epitope generation or for the ability of these phosphoproteins to regulate mitotic progression. Instead, precocious activation of Cdc25 may be the direct consequence of Pin1 removal.

Recently, it was reported that Pin1 antagonizes in vitro phosphorylation of the mitotic regulators Cdc25, Myt1, and Wee1 by Cdc2/cyclin B (*29*). Although the ability of Pin1 to bind mitotic phosphoproteins appears to be important for this inhibition, association alone is not sufficient for endogenous Pin1 function. We introduced a point mutation [$Cys^{109} \rightarrow Ala^{109}$ (C109A)] into *Xenopus*

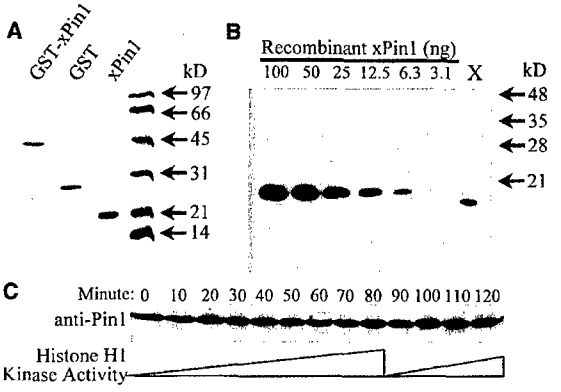

Fig 1. Identification of the *Xenopus laevis* Pin1 homolog. **(A)** Recombinant xPin1 was purified from bacteria as a glutathione *S*-transferase (GST)–xPin1 fusion protein and eluted by cleavage from the GST with thrombin. Coomassie blue staining of 1 μg of each indicated protein separated by SDS–polyacrylamide gel electrophoresis is shown. **(B)** Using antiserum to xPin1 (diluted 5000-fold), we recognized a single protein in *Xenopus* egg extract; 0.5 μl of extract was loaded in lane X. **(C)** The Pin1 content in *Xenopus* extracts (1 μl extract per lane) was visualized by immunoblot over the course of two cell cycles, as assessed by histone H1 kinase activity (*35*) and indicated schematically below the gel lanes.

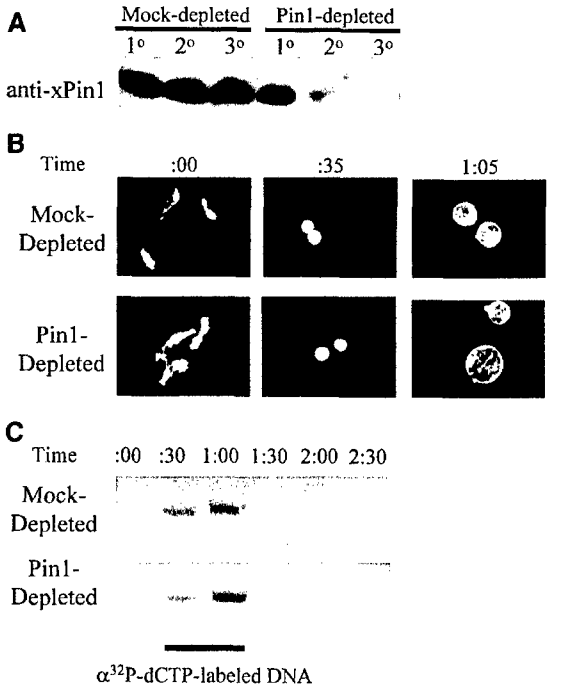

Fig. 2. Exit from mitosis in *Xenopus* extracts lacking Pin1. **(A)** Immunoblot of Pin1 remaining in CSF extracts (*33, 36*) after each stage of immunodepletion. Three successive treatments (1°, 2°, and 3°) removed >95% of Pin1 (*20*). **(B)** 3° depleted extracts were supplemented with demembranated sperm chromatin (*37*) and adenosine 5′-triphosphate (ATP) regenerating mix (*32*) and released from CSF arrest with 400 μM $CaCl_2$. At various times, measured with respect to the time of $CaCl_2$ addition in hours and minutes, portions of the extract were withdrawn, diluted 1:1 with Hoechst 33258 [10 μg/ml in 26% formaldehyde, 0.2 M sucrose, and 10 mM Hepes (pH 8.0)], and examined by fluorescence microscopy. **(C)** DNA replication was detected by pulse labeling of DNA in extracts with α^{32}P–deoxycytidine 5′-triphosphate, agarose gel electrophoresis, and autoradiography, as described (*22*).

Pin1 that compromised the prolyl isomerase activity of the enzyme by >90% (*30*) without diminishing its protein binding avidity (Fig. 4D). This mutant was incapable of restoring the checkpoint response in Pin1-depleted extracts when added to achieve concentrations sufficient for complementation of the checkpoint defect by wild-type xPin1 (Fig. 4B). Furthermore, *Xenopus* Pin1 complements the

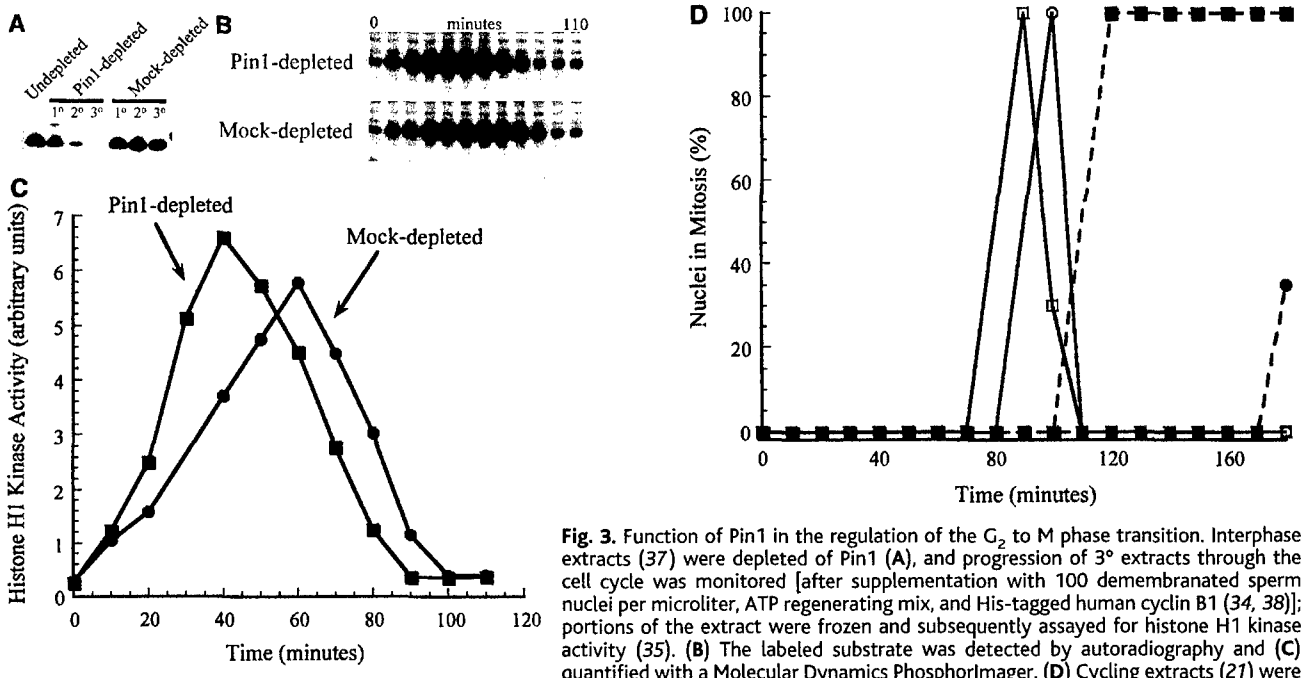

Fig. 3. Function of Pin1 in the regulation of the G_2 to M phase transition. Interphase extracts (*37*) were depleted of Pin1 (**A**), and progression of 3° extracts through the cell cycle was monitored [after supplementation with 100 demembranated sperm nuclei per microliter, ATP regenerating mix, and His-tagged human cyclin B1 (*34, 38*)]; portions of the extract were frozen and subsequently assayed for histone H1 kinase activity (*35*). (**B**) The labeled substrate was detected by autoradiography and (**C**) quantified with a Molecular Dynamics PhosphorImager. (**D**) Cycling extracts (*21*) were depleted of Pin1 (squares) or mock depleted (circles) and supplemented with ATP regenerating mix and 100 demembranated sperm nuclei per microliter (open symbols) or 500 nuclei per microliter (solid symbols). The mitotic index was monitored by fluorescence microscopy. Each of these experiments is representative of our observations in several extracts.

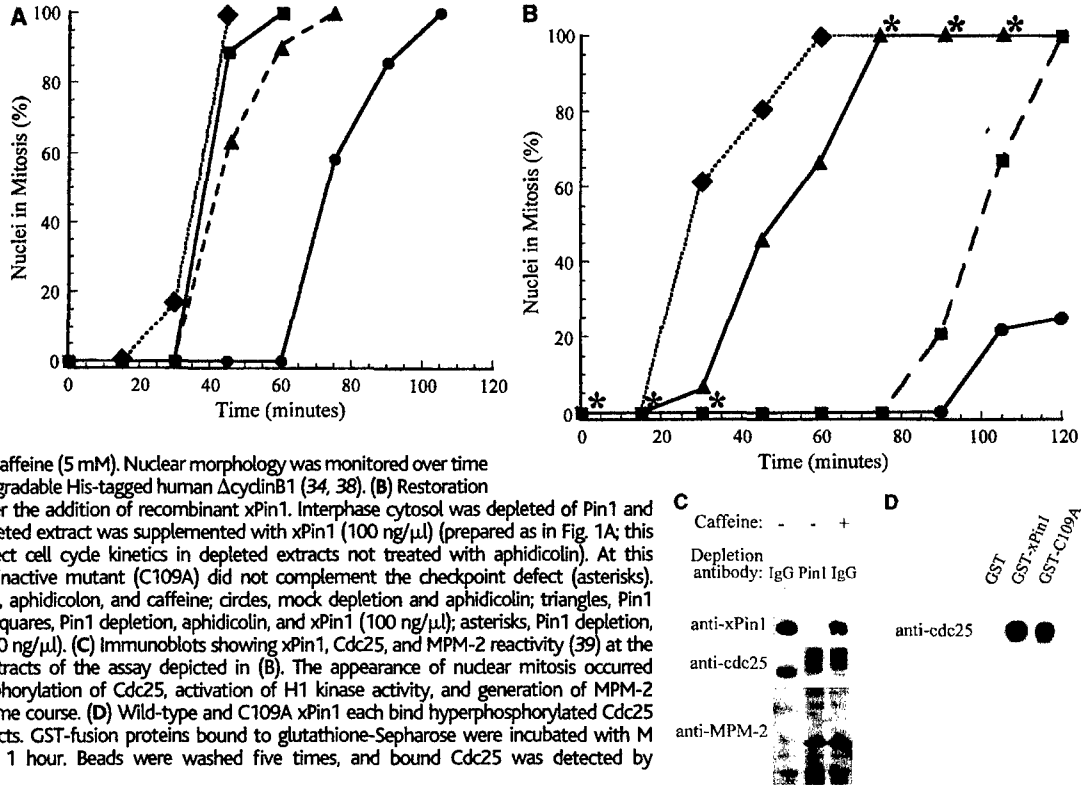

Fig. 4. Requirement of Pin1 for the checkpoint arrest in response to unreplicated DNA. (**A**) Depleted interphase cytosol (*37*) was supplemented with membranes isolated from cells in interphase (1:10), demembranated sperm nuclei (200 nuclei per microliter), and ATP regenerating mix. Dotted line with triangles, mock depletion; circles, mock depletion and aphidicolin (50 μg/μl); squares, Pin1 depletion and aphidicolin; dashed line with triangles, mock depletion, aphidicolin, and caffeine (5 mM). Nuclear morphology was monitored over time after the addition of nondegradable His-tagged human ΔcyclinB1 (*34, 38*). (**B**) Restoration of checkpoint function after the addition of recombinant xPin1. Interphase cytosol was depleted of Pin1 and treated as in (A). Pin1-depleted extract was supplemented with xPin1 (100 ng/μl) (prepared as in Fig. 1A; this concentration did not affect cell cycle kinetics in depleted extracts not treated with aphidicolin). At this concentration, the PPIase-inactive mutant (C109A) did not complement the checkpoint defect (asterisks). Diamonds, mock depletion, aphidicolon, and caffeine; circles, mock depletion and aphidicolin; triangles, Pin1 depletion and aphidicolin; squares, Pin1 depletion, aphidicolin, and xPin1 (100 ng/μl); asterisks, Pin1 depletion, aphidicolin, and C109A (100 ng/μl). (**C**) Immunoblots showing xPin1, Cdc25, and MPM-2 reactivity (*39*) at the 120-min time point in extracts of the assay depicted in (B). The appearance of nuclear mitosis occurred simultaneously with phosphorylation of Cdc25, activation of H1 kinase activity, and generation of MPM-2 epitopes throughout the time course. (**D**) Wild-type and C109A xPin1 each bind hyperphosphorylated Cdc25 in M phase *Xenopus* extracts. GST-fusion proteins bound to glutathione-Sepharose were incubated with M phase cytosol at 4°C for 1 hour. Beads were washed five times, and bound Cdc25 was detected by immunoblotting.

lethality of *ess1* mutants in budding yeast, but the C109A mutant is inactive in the complementation assay (*31*). Together, these observations indicate that Pin1 is functionally conserved and that its catalytic activity is required for its checkpoint role in *Xenopus* and its essential function in *S. cerevisiae*.

Pin1 participates in the replication checkpoint in a manner requiring its catalytic activity. Target-specific inhibition of mitosis-promoting kinase activity provides a biochemical mechanism for Pin1's role in enabling the replication checkpoint. Pin1 could mediate inhibition of Cdc25 hyperphosphorylation and act in concert with 14-3-3–mediated cytoplasmic sequestration of the phosphatase to prolong the G_2 phase by preventing functional interaction of Cdc25 with Cdc2 under checkpoint conditions. This function is consistent with previous observations that the G_2 phase is prolonged when Pin1 is overexpressed. In the absence of DNA perturbation, consequences of Pin1 removal may not be manifested; this would explain the lack of apparent phenotype in Pin1-null metazoans. Our demonstration of the essential role of Pin1 in the replication checkpoint establishes a position for endogenous Pin1 in the eukaryotic cell cycle regulatory network.

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39. The Cdc25 antibody was a gift from E. Shibuya. The MPM-2 antibody was purchased from Upstate Biotechnology (Lake Placid, NY).

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Requirement of the Inositol Trisphosphate Receptor for Activation of Store-Operated Ca^{2+} Channels

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The coupling mechanism between endoplasmic reticulum (ER) calcium ion (Ca^{2+}) stores and plasma membrane (PM) store-operated channels (SOCs) is crucial to Ca^{2+} signaling but has eluded detection. SOCs may be functionally related to the TRP family of receptor-operated channels. Direct comparison of endogenous SOCs with stably expressed TRP3 channels in human embryonic kidney (HEK293) cells revealed that TRP3 channels differ in being store independent. However, condensed cortical F-actin prevented activation of both SOC and TRP3 channels, which suggests that ER-PM interactions underlie coupling of both channels. A cell-permeant inhibitor of inositol trisphosphate receptor ($InsP_3R$) function, 2-aminoethoxydiphenyl borate, prevented both receptor-induced TRP3 activation and store-induced SOC activation. It is concluded that $InsP_3Rs$ mediate both SOC and TRP channel opening and that the $InsP_3R$ is essential for maintaining coupling between store emptying and physiological activation of SOCs.

Receptor-induced Ca^{2+} signals comprise two interdependent components—rapid Ca^{2+} release from Ca^{2+} stores in the ER and Ca^{2+} entry through slowly activating PM SOCs. The trigger for SOC activation is decreased Ca^{2+} in the ER lumen (*1, 2*). However, despite intense study, the ER-derived signal coupling store depletion with SOC activation remains unknown (*3*). Direct coupling between ER and PM has been hypothesized (*4, 5*), and evidence indicates that physical docking of ER with the PM is involved in SOC activation (*6–8*). The mammalian TRP family of receptor-operated ion channels has been suggested to share some operational

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Appendix B

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Maintenance of G_2 arrest in the *Xenopus* oocyte: a role for 14-3-3-mediated inhibition of Cdc25 nuclear import

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Cdc2–cyclin B1 in the G_2-arrested *Xenopus* oocyte is held inactive by phosphorylation of Cdc2 at two negative regulatory sites, Thr14 and Tyr15. Upon treatment with progesterone, these sites are dephosphorylated by the dual specificity phosphatase, Cdc25, leading to Cdc2–cyclin B1 activation. Whereas maintenance of the G_2 arrest depends upon preventing Cdc25-induced Cdc2 dephosphorylation, the mechanisms responsible for keeping Cdc25 in check in these cells have not yet been described. Here we report that Cdc25 in the G_2-arrested oocyte is bound to 14-3-3 proteins and that progesterone treatment abrogates this binding. We demonstrate that Cdc25, apparently statically localized in the cytoplasm, is actually capable of shuttling in and out of the oocyte nucleus. Binding of 14-3-3 protein markedly reduces the nuclear import rate of Cdc25, allowing nuclear export mediated by a nuclear export sequence present in the N-terminus of Cdc25 to predominate. If 14-3-3 binding to Cdc25 is prevented while nuclear export is inhibited, the coordinate nuclear accumulation of Cdc25 and Cdc2–cyclin B1 facilitates their mutual activation, thereby promoting oocyte maturation.

Keywords: 14-3-3 protein/Cdc2–cyclin B1/Cdc25/oocyte maturation/*Xenopus*

Introduction

Xenopus oocytes are physiologically arrested in G_2 of meiosis I. Upon treatment with progesterone, these oocytes undergo meiotic maturation, leading to breakdown of the nuclear envelope (germinal vesicle breakdown; GVBD), chromosome condensation and spindle formation. Whereas many of the molecular details of this process have yet to be elucidated, it is clear that obligatory steps in progesterone-induced oocyte maturation include translation of mRNA encoding the mos protein kinase, consequent activation of a mitogen-activated protein kinase (MAPK) cascade and activation of maturation promoting factor (MPF), consisting of a Cdc2 kinase catalytic subunit complexed to a B-type cyclin (Masui and Markert, 1971; Sagata *et al.*, 1989; Kanki and Donoghue, 1991; Nebreda and Hunt, 1993; Shibuya and Ruderman, 1993; Kosako *et al.*, 1994).

In the oocyte, Cdc2–cyclin B complexes are stockpiled in an inactive form, poised for progesterone-induced activation. Phosphorylation of the Cdc2 subunit at two negative regulatory sites, Thr14 and Tyr15, is primarily responsible for the inactivity of the stored complexes (Cyert and Kirschner, 1988; Gautier and Maller, 1991; Kobayashi *et al.*, 1991). In somatic cells, phosphorylation of Tyr15 is catalyzed by two related kinases: a nuclear kinase called Wee1, and a cytoplasmic, membrane-associated kinase called Myt1 [Myt1 also phosphorylates Thr14; reviewed in Coleman and Dunphy (1994) and Lew and Kornbluth (1996)]. However, Wee1 appears to be entirely absent from *Xenopus* oocytes, so Myt1 is thought to be primarily responsible for phosphorylating Cdc2 in these cells (Murakami and Vande Woude, 1998). It has recently been reported that progesterone treatment leads to inactivation of Myt1, through a MAPK-induced activation of the kinase $p90^{rsk}$ (Palmer *et al.*, 1998). Myt1 physically associates with the active hyperphosphorylated form of rsk and phosphorylation of Myt1 by rsk inhibits Myt1 activity. Presumably, inactivation of Myt1 allows the dephosphorylation of Cdc2 Thr14 and Tyr15, leading to MPF activation (Atherton-Fessler *et al.*, 1994; Kornbluth *et al.*, 1994; Mueller *et al.*, 1995). Dephosphorylation of Thr14 and Tyr15 on Cdc2 is catalyzed by the dual (Thr/Tyr) specificity phosphatase, Cdc25 (Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Strausfeld *et al.*, 1991; Millar and Russell, 1992). In somatic cells, Cdc25 alternates between an interphase form with low activity and a hyperphosphorylated mitotic form with high activity. Oocyte maturation is also accompanied by Cdc25 hyperphosphorylation (as evidenced by a shift in the electrophoretic mobility of Cdc25) and activation (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Hoffmann *et al.*, 1993).

Although physiologically distinct from the G_2 arrest of the oocyte, the checkpoint-induced G_2 arrest of somatic cells in response to DNA damage or stalled DNA replication also involves the suppression of pre-formed Cdc2–cyclin B complexes through phosphorylation of Cdc2 at Thr14 and Tyr15 (Enoch and Nurse, 1990; Enoch *et al.*, 1992; Smythe and Newport, 1992). Several groups have recently demonstrated that checkpoint-activated kinases phosphorylate Cdc25 at a critical regulatory site (Ser216 of human Cdc25C or Ser287 of *Xenopus* Cdc25; Peng *et al.*, 1997; Kumagai *et al.*, 1998a; Zeng *et al.*, 1998). This phosphorylation creates a binding site for members of a family of small acidic proteins collectively called 14-3-3 proteins (Peng *et al.*, 1997; Kumagai *et al.*, 1998b; Zeng *et al.*, 1998). Binding by 14-3-3 seems to functionally 'inactivate' Cdc25, and is critical for maintaining the checkpoint-induced G_2 arrest. Interestingly, 14-3-3 binding does not alter Cdc25 activity assayed *in vitro*, suggesting that 14-3-3 somehow sequesters Cdc25, perhaps altering its subcellular localization to prevent access of Cdc25 to the Cdc2–cyclin B substrates (Peng *et al.*, 1997).

Factors which modulate the subcellular localization of Cdc2–cyclin B complexes may also contribute to the maintenance of DNA-responsive checkpoint-induced cell-cycle arrest (Jin *et al.*, 1998; Toyoshima *et al.*, 1998). We and others have recently shown that Cdc2–cyclin B1 complexes, which appear to be statically localized to the cytoplasm during interphase, actually shuttle continuously in and out of the nucleus, where they might in some way 'read' the status of the DNA (Hagting *et al.*, 1998; Toyoshima *et al.*, 1998; Yang *et al.*, 1998). During interphase, nuclear export of cyclin B1, mediated by the nuclear export receptor CRM1, predominates over nuclear import. However, at the G_2/M transition, phosphorylation of cyclin B1 in the region of its nuclear export sequence (NES) prevents nuclear export, thereby fostering the nuclear accumulation of Cdc2–cyclin B1 required for the nuclear events of mitosis (Li *et al.*, 1995, 1997; Yang *et al.*, 1998). Although it has not been demonstrated that the DNA damage or replication checkpoints directly regulate cyclin B localization, there are data to suggest that this may be the case. Indeed, forcibly localizing cyclin B1 to the nucleus by appending a strong nuclear localization sequence, or by inactivating its nuclear export, compromises checkpoint function (Jin *et al.*, 1998; Toyoshima *et al.*, 1998).

In this report, we show that Cdc25 in the G_2-arrested *Xenopus* oocyte is complexed to 14-3-3 proteins, and that this binding is abrogated by progesterone treatment. Further, we demonstrate that the apparently cytoplasmic *Xenopus* Cdc25 contains an intrinsic CRM1-binding nuclear export sequence and can, like cyclin B1, shuttle in and out of the nucleus. Mutation of Cdc25 to prevent 14-3-3 binding resulted in a dramatic increase in the nuclear import rate of Cdc25, without markedly perturbing its nuclear export rate. These findings indicate that the G_2 arrest of the oocyte employs similar strategies to those operating in response to checkpoint controls, and provides a mechanistic basis for the functional inhibition of Cdc25 by 14-3-3 proteins.

Results

To determine whether *Xenopus* Cdc25 bound 14-3-3 proteins in the G_2-arrested oocyte, we prepared extracts from either untreated oocytes or oocytes after progesterone treatment. When anti-Cdc25 immunoprecipitates from these extracts were immunoblotted with antisera directed against 14-3-3 ε, the predominant Cdc25-binding variant in interphase *Xenopus* egg extracts (Kumagai *et al.*, 1998), we found that the G_2-arrested oocytes contained Cdc25–14-3-3 complexes, which were no longer detectable at the time of progesterone-induced GVBD (Figure 1). These data suggested the possibility that Cdc25 binding by 14-3-3 proteins might contribute to the maintenance of G_2 arrest in the oocyte.

Cytoplasmic accumulation of Cdc25 reflects continuous nuclear import and rapid re-export

Since binding of Cdc25 by 14-3-3 proteins does not appear to alter Cdc25 enzymatic activity, we wished to explore the possibility that 14-3-3 binding might regulate the subcellular localization of Cdc25 in the oocyte. Like cyclin B1, Cdc25C is cytoplasmic during interphase and enters the nucleus just prior to mitosis, at least in some somatic cells (Seki *et al.*, 1992). Also like cyclin B1, Cdc25 is almost entirely cytoplasmic in the G_2-arrested *Xenopus* oocyte (Izumi *et al.*, 1992; Yang *et al.*, 1998). We have recently shown that cyclin B1 in fact shuttles in and out of the germinal vesicle (GV), leading us to suspect that the apparently static localization of Cdc25 belied its ability to shuttle in and out of nuclei. To test this possibility, we monitored Cdc25 localization after treating oocytes with leptomycin B, an inhibitor of CRM1-mediated nuclear export (Fornerod *et al.*, 1997; Neville *et al.*, 1997; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997; Wolff *et al.*, 1997). Anti-Cdc25 immunoblotting of nuclear and cytoplasmic fractions from manually dissected oocytes revealed that inhibiting nuclear export promoted a striking nuclear accumulation of Cdc25, which occurred even faster than cyclin B1 nuclear accumulation in the same system (Figure 2A and B; Yang *et al.*, 1998). Thus, as for cyclin B1, cytoplasmic localization of Cdc25 results from ongoing nuclear import and more rapid re-export.

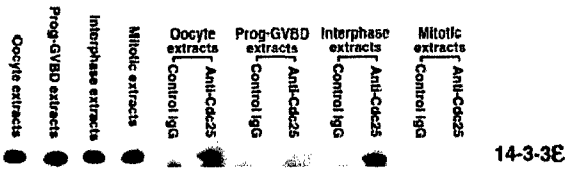

Fig. 1. *Xenopus* Cdc25 binds to 14-3-3 ε in oocyte extracts and interphase egg extracts. Extracts were prepared from untreated *Xenopus* oocytes, oocytes undergoing progesterone-induced GVBD, *Xenopus* eggs arrested in mitosis or *Xenopus* eggs released into interphase. Two microliters of each extract was subjected to SDS–PAGE and immunoblotted with anti-14-3-3 ε antibody (first four lanes). In addition, 60 μl of each extract was immunoprecipitated with either control IgG or anti-Cdc25 sera. Immunoprecipitated proteins were analyzed by SDS–PAGE followed by Western blotting with anti-14-3-3 ε antibody. 14-3-3 ε coprecipitated with Cdc25 only in the untreated oocyte and interphase egg extracts.

Xenopus Cdc25 is a nuclear export substrate in both the presence and absence of 14-3-3 binding

To determine if 14-3-3 binding affected Cdc25 localization, we wished to examine the consequences of this binding for Cdc25 nuclear import and export rates. To confirm that Cdc25 could, indeed, serve as a nuclear export substrate, we injected ^{35}S-labeled *in vitro*-translated Cdc25 into oocyte nuclei, and then manually dissected the oocytes into cytoplasmic and nuclear fractions at various times after injection. Within 60 min, virtually all of the Cdc25 had been exported from nuclei (Figure 2C). When we injected nuclei from the same batch of oocytes with Cdc25 protein which had been mutated to abrogate 14-3-3 binding (Cdc25 S287A), we found that the mutant Cdc25 appeared to exit nuclei at a rate only marginally slower than the wild-type (a point we will return to below).

Identification of a CRM1-binding NES in Cdc25

The inhibition of Cdc25 nuclear export by leptomycin-B suggested that Cdc25 export was mediated by CRM1. We produced recombinant derivatives of Cdc25 in *Escherichia coli* to examine whether they could bind to CRM1 in oocyte extracts. First, we fused only the N-terminal 322 amino acids or the C-terminal 228 amino acids of Cdc25 to glutathione *S*-transferase (GST), immobilized

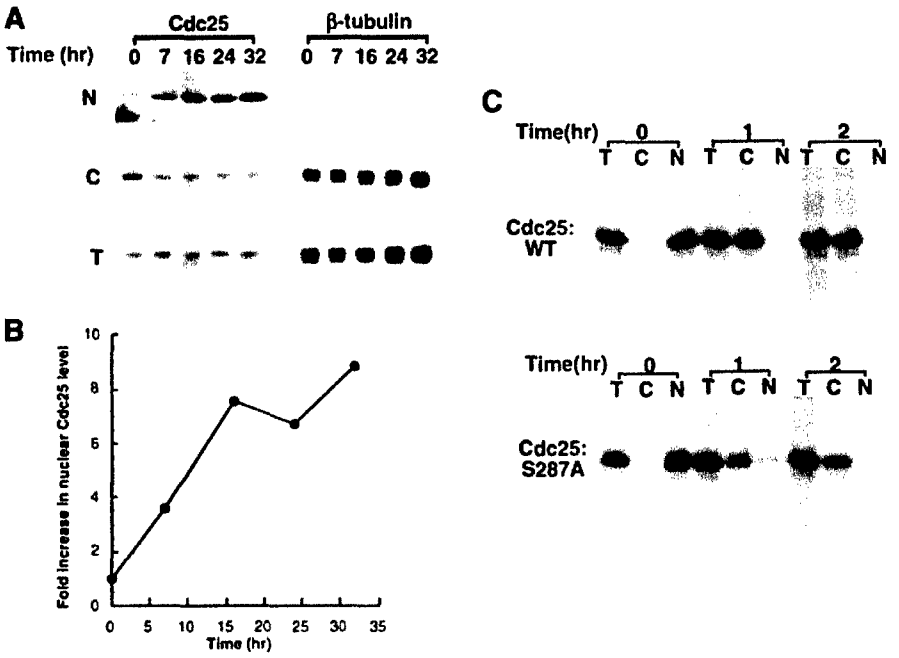

Fig. 2. Leptomycin B treatment induces endogenous Cdc25 nuclear accumulation in *Xenopus* oocytes and *Xenopus* Cdc25 can be exported from nuclei both in the presence and absence of 14-3-3 binding. (**A**) Oocytes were treated with 200 nM leptomycin B in MB buffer. At the times indicated after treatment, they were dissected into cytoplasmic and nuclear fractions. Proteins were extracted and analyzed by SDS–PAGE followed by immunoblotting with anti-*Xenopus* Cdc25 antibody. The samples were also blotted with anti-β-tubulin antibody as a control for dissection and loading. T, total; C, cytoplasmic fraction; N, nuclear fraction. For every oocyte nuclear equivalent loaded onto the gel, only 1/8 the amount of cytoplasm was loaded to facilitate observation of potential changes in cytoplasmic levels of the protein. For every oocyte nuclear equivalent loaded on the gel, 1/8 of a total oocyte was loaded in the 'T' lane. (**B**) The graph represents a quantitation of the data in (A), showing the fold increase of endogenous Cdc25 accumulated in nuclei at the times indicated after leptomycin B treatment. (**C**) Wild-type or S287A mutant Cdc25 protein was translated in reticulocyte lysates in the presence of ^{35}S-methionine and injected into *Xenopus* oocytes. At 0, 1 and 2 h after injection, the oocytes were dissected and successfully injected oocytes were identified by the presence of pink coloring from the reticulocyte lysate. These oocytes were separated into cytoplasmic and nuclear fractions, extracted and analyzed by SDS–PAGE followed by autoradiography.

the fusion proteins on glutathione–Sepharose and incubated these in oocyte extract as a source of CRM1. After extensive washing, proteins remaining bound to the beads were resolved by SDS–PAGE and immunoblotted with CRM1 antisera. As shown in Figure 3A, a protein containing the N-terminal 322 amino acids of Cdc25 was able to bind CRM1, whereas a protein containing the C-terminal 228 amino acids of Cdc25 was not. Furthermore, a Cdc25 N-terminal fragment containing the S287A mutation bound equally well to CRM1 despite its inability to bind 14-3-3 (Figure 3B). This suggests that the Cdc25–CRM1 interaction is unaffected by 14-3-3, consistent with the observed similar export rates of the full-length wild-type and S287A mutant Cdc25 proteins.

To localize the Cdc25 NES more precisely, we fused GST to successively smaller portions of the N-terminal fragment, serially deleting regions of the protein, starting from its C-terminus (aa 322; Figure 3C). As above, these fusion proteins were tested for their ability to retrieve CRM1 from oocyte extracts. We found that all of the fusion proteins examined, including one containing only the first 100 amino acids of Cdc25, were able to interact with CRM1 (Figure 3D). A scan of the first 100 amino acids revealed a single sequence, 47LTPVTDLAV55, matching the consensus sequence for a CRM1-binding, leucine-rich NES (Bogerd *et al.*, 1996). To determine whether this sequence affected nuclear export of Cdc25, we mutated the last two required hydrophobic residues, L53 and V55, to Ala (Figure 4A). This mutant protein was not detectably exported following microinjection into oocyte nuclei (Figure 4B and C) and a recombinant truncated Cdc25 protein bearing the same mutations bound well to 14-3-3 protein, but could not bind to CRM1 (Figure 4D). These data strongly suggest that the mutated residues lie within a functional NES which is responsible for *Xenopus* Cdc25 nuclear export.

Cdc25 S287A induces GVBD in leptomycin-B-treated oocytes

Since 14-3-3 binding did not appear to modulate the nuclear export rate of Cdc25, we wished to determine whether the nuclear import rate of Cdc25 might be affected. To this end, we injected radiolabeled wild-type or S287A Cdc25 proteins into the cytoplasm of oocytes which had been pre-treated with leptomycin B to prevent re-export. Intriguingly, under these conditions the S287A protein induced MPF activation (assayed as histone H1-directed kinase activity) and GVBD ~6 h after injection (Figure 5A). This effect required leptomycin B treatment and did not occur with the wild-type Cdc25. Since we injected trace quantities of radiolabeled protein (<2% of endogenous Cdc25), this result indicates that the S287A Cdc25 mutant has considerably increased biological potency compared with the wild-type, in agreement with the recently described relative potency of similar Cdc25 mutants in overcoming a checkpoint arrest in somatic

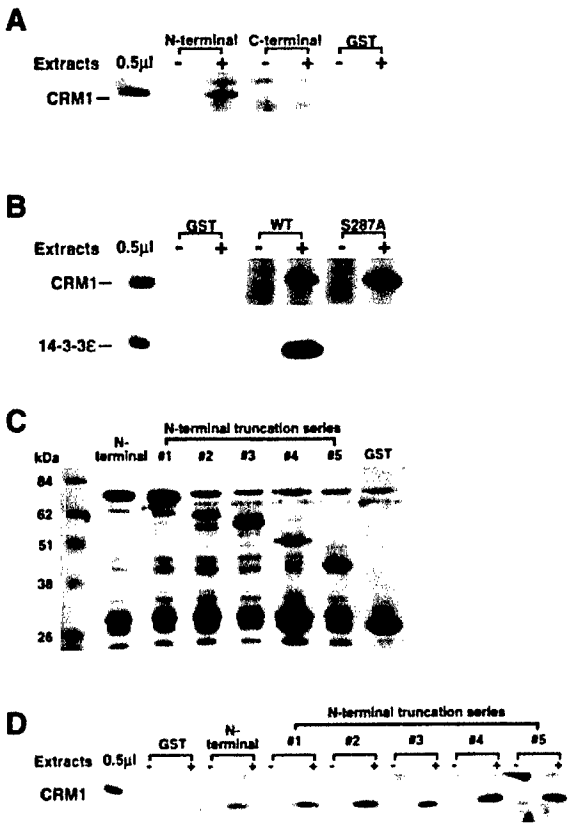

Fig. 3. The N-terminal region of *Xenopus* Cdc25 contains a CRM1-binding NES, which can bind to CRM1 in the presence and absence of 14-3-3 binding. (**A**) N-terminal (aa. 1–322) or C-terminal (aa. 323–550) fragments of Cdc25 were fused to GST and coupled to glutathione–Sepharose beads. Twenty microliters of these resins or control GST resin were incubated in 100 µl of interphase extract for 1 h. The beads were then pelleted and washed five times with egg lysis buffer. The bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting with anti-human CRM1 antibody. (**B**) The N-terminal (aa 1–322) fragments of wild-type or S287A mutant Cdc25 proteins were fused to GST and coupled to glutathione–Sepharose beads. The pull-down assay was performed as in (A), and the bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting with anti-CRM1 and anti-14-3-3 ε antibody. (**C**) The truncated proteins derived from the N-terminal fragment of Cdc25 were fused to GST and coupled to glutathione–Sepharose beads. The bead-bound proteins were boiled in SDS sample buffer and eluted proteins were subjected to SDS–PAGE and Coomassie-blue staining, to show the molecular weights of the deleted fusion proteins. (**D**) Twenty microliters of the resins shown in (C) were used for pull-down assays as described in (A). The bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting with anti-human CRM1 antibody.

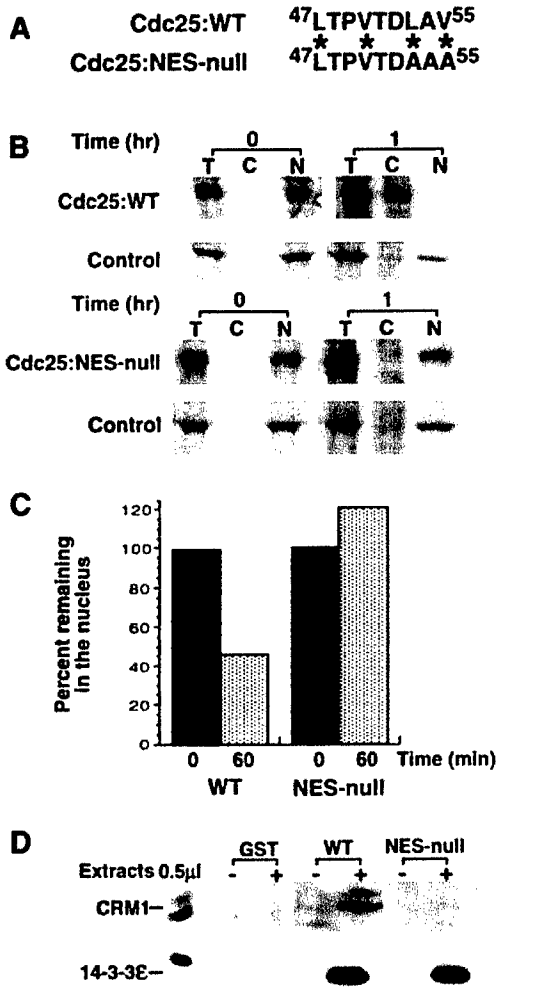

Fig. 4. Identification of the CRM1-binding NES in *Xenopus* Cdc25. (**A**) Residues 47–55 of *Xenopus* Cdc25 comprise a putative NES. Asterisks indicate consensus leucine-rich residues. L53 and V55 were mutated to Ala to create the Cdc25:NES-null protein. (**B**) ^{35}S-labeled Cdc25:WT or Cdc25:NES-null proteins were coinjected into oocyte nuclei with GRP94 control protein. Oocytes were dissected 0 or 1 h later and proteins were extracted and analyzed by SDS–PAGE and autoradiography. By 2 h (not shown), the NES-null protein had still not exited from nuclei. (**C**) The bar graph represents a quantitation of the data in (B), showing the percentage of Cdc25 remaining in nuclei after 0 and 1 h. Values were normalized to coinjected GRP94 protein. (**D**) The N-terminal (aa. 1–322) fragment of wild-type or NES-null mutant Cdc25 proteins were fused to GST and coupled to glutathione–Sepharose beads. The pull-down assay was performed as in Figure 3A, and the bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting with anti-CRM1 and anti-14-3-3 ε antibody.

cells (Kumagai *et al.*, 1998b; Peng *et al.*, 1997; Zeng *et al.*, 1998).

Intra-nuclear activation of Cdc25 S287A by Cdc2–cyclin B

In the above experiments, GVBD was preceded by nuclear accumulation and hyperphosphorylation of the S287A Cdc25, detected by an electrophoretic mobility shift upon SDS–PAGE (Figure 5B). A similar mobility shift of Cdc25 has been reported in response to phosphorylation by active Cdc2–cyclin B and by the kinase Plx1 (Kumagai and Dunphy, 1992, 1996; Hoffmann *et al.*, 1993; Izumi and Maller, 1995). In both cases, Cdc25 hyperphosphorylation correlates with an increase in its enzymatic activity. Since leptomycin B treatment also induces nuclear accumulation of Cdc2–cyclin B1, we wished to determine whether Cdc2–cyclin B might be responsible for the hyperphosphorylation of S287A Cdc25 in leptomycin-B-treated oocytes. Therefore, we repeated the wild-type and mutant Cdc25 injection experiments using oocytes which had been pre-incubated with both leptomycin B and the Cdc2 inhibitor, roscovitine (50 µM; Meijer *et al.*, 1997). Roscovitine effectively eliminated the S287A Cdc25 mobility shift (and GVBD), consistent with a role for

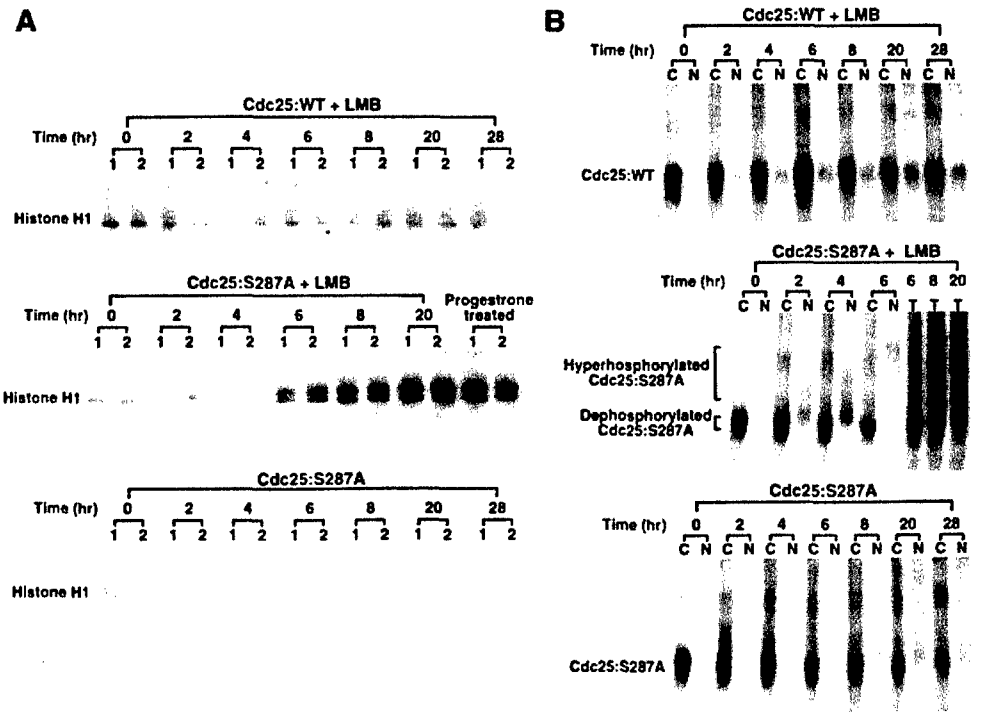

Fig. 5. Cdc25:S287A protein induces GVBD in leptomycin B-treated oocytes and is hyperphosphorylated in these oocyte nuclei. (**A**) Oocytes were incubated with or without 200 nM leptomycin B in MB buffer for 2 h before injection. Forty nanoliters of ^{35}S-labeled Cdc25:WT or S287A protein in reticulocyte lysate was coinjected into oocyte cytoplasm with ^{14}C-labeled BSA as control. Two oocytes were collected at each of the indicated times after injection and immediately frozen in liquid nitrogen. The samples were assayed for Cdc2 kinase activity using histone H1 as a substrate. Progesterone-treated oocytes, which had already undergone GVBD, were used as a positive control. (**B**) Oocytes treated and injected as described in (A), oocytes were dissected into cytoplasmic and nuclear fractions at the indicated times after injection and proteins were analyzed by SDS–PAGE and autoradiography. For leptomycin-B-treated oocytes injected with S287A Cdc25, oocytes started GVBD at 6 h and had all undergone GVBD by 8 h. For oocytes injected with wild-type Cdc25 or not treated with leptomycin B, GVBD had not occurred even 28 h after injection.

Cdc2–cyclin B in the observed Cdc25 hyperphosphorylation (Figure 6A). At similar concentrations, roscovitine did not prevent Cdc25 hyperphosphorylation by kinases active in interphase egg extracts treated with the phosphatase inhibitor, microcystin (5 μM; Figure 6B). These egg extracts lack cyclins A and B (and hence lack active Cdc2), but are induced to enter a pseudomitotic state by incubation with microcystin and contain a variety of activated non-Cdc2 kinases including Plx1 (Izumi and Maller, 1995; Kumagai and Dunphy, 1996; Qian *et al.*, 1998). Thus, the lack of inhibition of these non-Cdc2 kinases by roscovitine supports its reported specificity as an inhibitor and suggests that Cdc2–cyclin B was, indeed, responsible for phosphorylating and activating S287A Cdc25 in the oocyte. In aggregate, these data suggest that abrogating the Cdc25–14-3-3 interaction with the S287A mutation creates a more potent Cdc25 that triggers a feedback loop involving the mutual activation of Cdc25 and Cdc2/cyclin-B in the nuclei of leptomycin-B-treated oocytes.

The S287A mutant of Cdc25 is imported into nuclei more efficiently than the wild-type protein

What is the basis for the increased potency of S287A Cdc25? Since both Cdc2–cyclin B1 and Cdc25 gradually accumulate in oocyte nuclei upon inhibition of nuclear export with leptomycin B, one possibility is that the S287A mutation increases the nuclear import rate of Cdc25. Faster accumulation of the trace amount of injected Cdc25 in the nucleus (away from the countervailing inhibitory action of Myt1 on Cdc2–cyclin B) may allow it to trigger the activating feedback loop leading to GVBD. To compare the nuclear import rates of wild-type and S287A Cdc25 proteins, we repeated the cytoplasmic injection experiments, but used roscovitine to prevent GVBD and permit analysis of nuclear import (in leptomycin-B-treated oocytes, to prevent Cdc25 re-export). At various times after injection, oocytes were separated into cytoplasmic and nuclear fractions and resolved by SDS–PAGE. As shown in Figure 7, the S287A mutant protein accumulated in nuclei at a markedly faster rate than the wild-type protein. These data strongly suggest that S287 phosphorylation, and consequent 14-3-3 binding, reduces Cdc25 nuclear import in the oocyte.

How does 14-3-3 binding affect nuclear import of Cdc25? Scanning of the *Xenopus* Cdc25 sequence revealed an evolutionarily conserved, consensus bipartite basic NLS, KR X_{13} KRRR at amino acids 298–316. Consistent with its containing a classical NLS, the N-terminal 322 amino acids of Cdc25 bound to the nuclear import receptor for such sequences, importin-α/β (Figure 8A; Gorlich *et al.*, 1994, 1995). Mutation of residues 313–315 of the candidate NLS to Ala severely impaired nuclear import of the mutant Cdc25 (Figure 8B) and greatly reduced binding to the importin-α/β heterodimer (Figure 8A). Consistent with its enhanced rate of nuclear import, the S287A Cdc25 mutant bound significantly better than the wild-type Cdc25 protein to the importin-α/β heterodimer (Figure 9A and B).

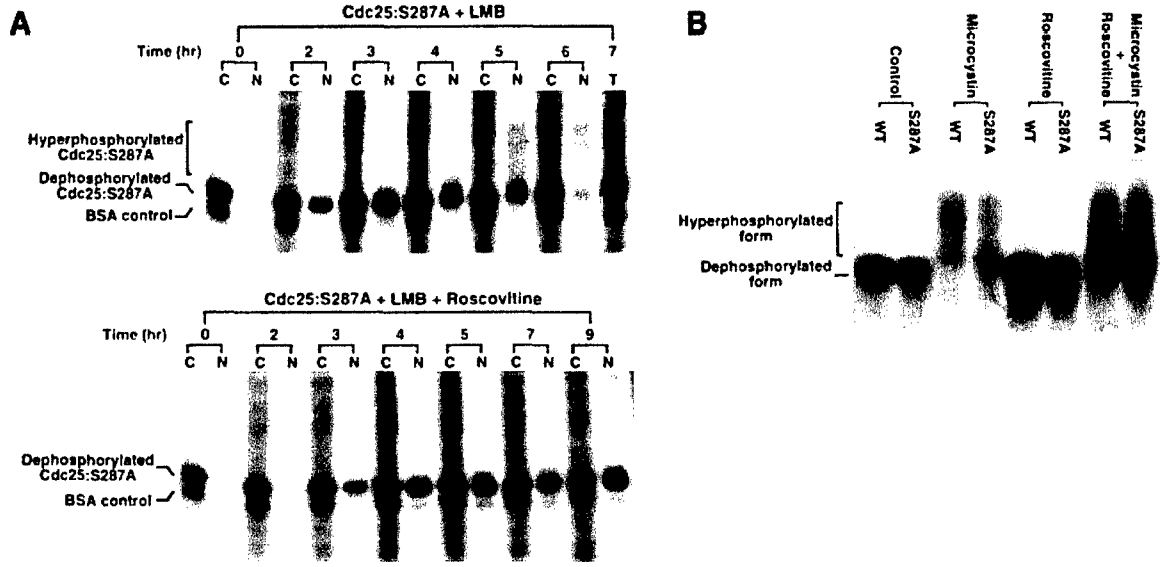

Fig. 6. Cdc2–cyclin B is responsible for the hyperphosphorylation of Cdc25:S287A in leptomycin-B-treated oocyte nuclei. (**A**) Oocytes were incubated with either 200 nM leptomycin B in MB buffer for 2 h before injection, or with both 200 nM leptomycin B and 50 μM roscovitine. Forty nanoliters of ^{35}S-labeled S287A protein translated *in vitro* was coinjected into oocyte cytoplasm with ^{14}C-labeled BSA as control. Oocytes were dissected into cytoplasmic and nuclear fractions at the indicated times after injection and proteins were analyzed by SDS–PAGE followed by autoradiography. Oocytes not treated with roscovitine entered GVBD at 7 h, whereas those treated with roscovitine did not manifest GVBD even after incubation overnight. (**B**) Two microliters of ^{35}S-labeled wild-type or S287A mutant Cdc25 protein was added to 20 μl of interphase *Xenopus* egg extract. These extracts were incubated with 5 μM microcystine, 50 μM roscovitine or 5 μM microcystin and 50 μM roscovitine at room temperature for 30 min in the presence of 2 mM ATP. The samples were subjected to SDS–PAGE and autoradiography.

When we injected NLS-null Cdc25 or S287A, NLS-null doubly mutant Cdc25 into oocyte nuclei to compare wild-type and S287A export rates under conditions where re-import could not occur, we did not observe any significant differences in their rates of nuclear export (Figure 9C). This demonstrates that the slight difference in the export rates of wild-type and S287A Cdc25 proteins shown in Figure 2C was due to faster re-import of exported S287A Cdc25. These data strongly suggest that 14-3-3 binding to *Xenopus* Cdc25 in oocytes exerts effects on nuclear shuttling by altering the rate of Cdc25 nuclear import, rather than export. Moreover, they indicate that 14-3-3 binding controls Cdc25 biological activity, at least in part, by inhibiting its entry into the nucleus.

Discussion

Binding of 14-3-3 to Cdc25 in G_2-arrested oocytes

We have shown that Cdc25 in the G_2-arrested oocyte can be found in a complex with 14-3-3 proteins, and that this complex is dissociated following progesterone treatment. In somatic cells, Cdc25 is phosphorylated in response to DNA damage and DNA-replication-induced checkpoint activation (Furnari *et al.*, 1997; Peng *et al.*, 1997; Sanchez *et al.*, 1997). Recent studies have demonstrated that this phosphorylation (at residue S287 of *Xenopus* Cdc25) results in the formation of a 14-3-3–Cdc25 complex which is important for maintaining the checkpoint-induced G_2 arrest (Peng *et al.*, 1997; Kumagai *et al.*, 1998b; Zeng *et al.*, 1998). In *Xenopus* oocytes, S287 phosphorylation is also required for 14-3-3 binding. These parallels suggest that similar strategies are employed to maintain a G_2 arrest in oocytes and somatic cells in response to different physiological stimuli. It will be interesting to determine whether other pathways functioning in the oocyte, such as regulation of Myt1 activity by rsk, also contribute to mitotic control in somatic cells.

14-3-3 binding selectively inhibits nuclear import of Cdc25 in oocytes

The mechanism whereby 14-3-3 binding functionally suppresses Cdc25 activity has been mysterious. We have found that Cdc25 accumulates almost exclusively in the cytoplasm of the oocyte as a result of a steady-state situation in which Cdc25 slowly enters the nucleus and is rapidly re-exported back to the cytoplasm. Our data demonstrate that the rate of Cdc25 nuclear import is greatly accelerated by mutation of S287 to non-phosphorylatable Ala. This suggests that S287 phosphorylation, and consequent 14-3-3 binding, significantly reduce Cdc25 nuclear import. We have identified a functional and evolutionarily conserved NLS in Cdc25 that lies adjacent to the site of 14-3-3 binding. Hence, 14-3-3 binding may sterically block access of Cdc25 to the nuclear import machinery.

Export of Cdc25 from oocyte nuclei was inhibited by leptomycin B, suggesting the involvement of the export factor CRM1. Indeed, we found that an N-terminal fragment of Cdc25 containing a putative NES sequence could bind to CRM1. Mutagenesis experiments demonstrated that this sequence was critical for both CRM1 binding and for nuclear export of Cdc25, and therefore constituted a functional NES. Unlike its dramatic effect on the nuclear import of Cdc25, mutation of S287 to Ala had no significant effect on the rate of Cdc25 nuclear export. This was confirmed in export assays in which re-import of Cdc25 was eliminated by mutation of the Cdc25 NLS. Thus, binding of 14-3-3 selectively reduces the rate of

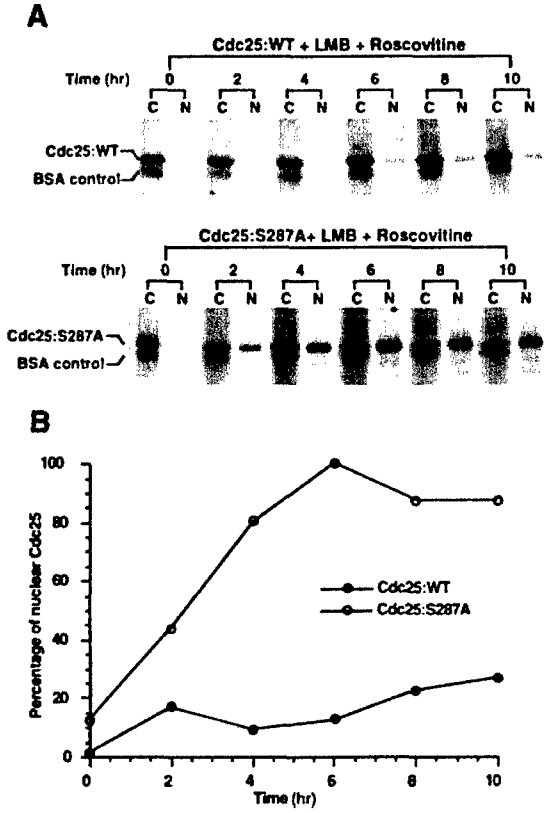

Fig. 7. The S287A mutant of Cdc25 is imported into nuclei more efficiently than the wild-type protein. (**A**) Oocytes were incubated with 200 nM leptomycin B and 50 μM roscovitine in MB buffer for 2 h before injection. Forty nanoliters of ^{35}S-labeled *in vitro* translated Cdc25:WT or S287A protein was injected into the cytoplasm of oocytes along with ^{14}C-labeled BSA as control. Injected oocytes were dissected into cytoplasmic and nuclear fractions at the indicated times after injection and proteins were analyzed by SDS–PAGE and autoradiography. (**B**) The graph represents a quantitation of the data in (A), showing the percentage of Cdc25 in the nuclear fraction at the indicated times.

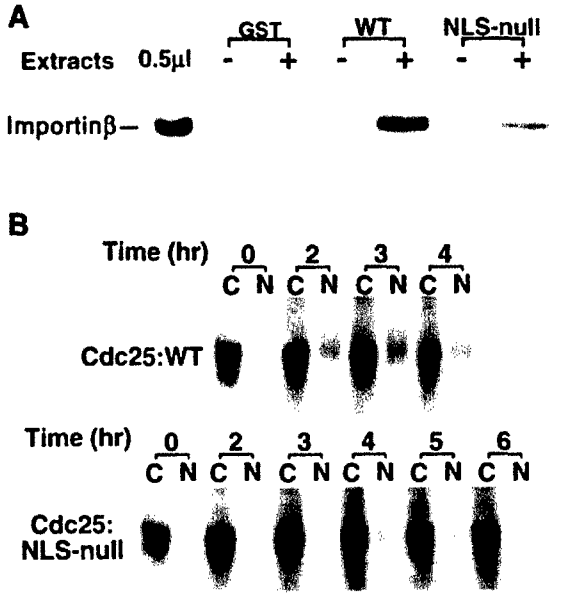

Fig. 8. Identification of a bipartite basic NLS in Cdc25. (**A**) The N-terminal (aa. 1–322) fragment of wild-type or NLS-null (K313A,R314A,R315A) mutant Cdc25 proteins were fused to GST and coupled to glutathione–Sepharose beads. Twenty microliters of these resins or control GST resin were incubated in 100 μl of interphase egg extract for 1 h. The beads were then pelleted and washed five times with egg lysis buffer. The bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting with anti-importin-β antibody. Note that classical NLSs bind importin-β via importin-α provided by the extract. (**B**) Forty nanoliters of ^{35}S-labeled *in vitro*-translated Cdc25:WT or Cdc25:NLS-null proteins were injected into the cytoplasm of oocytes. Injected oocytes were dissected into cytoplasmic and nuclear fractions at the times indicated after injection and proteins were analyzed by SDS–PAGE followed by autoradiography.

Cdc25 nuclear import while leaving its rate of export unaffected, presumably resulting in more efficient exclusion of Cdc25 from the nucleus.

Very recently, Lopez-Girona *et al.* (1999) reported studies in the fission yeast *Schizosaccharomyces pombe* which also indicate that 14-3-3 binding leads to exclusion of Cdc25 from the nucleus. However, this similar outcome was proposed to arise from a distinct mechanism, in which 14-3-3 binding provided a portable NES for Cdc25 nuclear export. This is clearly not the case in *Xenopus* oocytes, because non-phosphorylatable Cdc25 incapable of binding 14-3-3 is fully competent for export mediated by the Cdc25-intrinsic NES. Furthermore, a mutant Cdc25 lacking a functional NES was unable to be exported despite its continued ability to bind 14-3-3, suggesting that in this system 14-3-3 binding is neither necessary nor sufficient for Cdc25 nuclear export. These apparent differences between fission yeast and *Xenopus* may reflect the fact that the NES we have identified in *Xenopus* Cdc25 does not appear to be evolutionarily conserved. Thus, different cells may use different mechanisms for achieving nuclear exclusion of Cdc25 in response to 14-3-3 binding.

14-3-3 binding to Cdc25 collaborates with CRM1-mediated nuclear export of unknown factors to maintain the G_2 arrest in the oocyte

The importance of 14-3-3 binding for the suppression of Cdc25 biological activity was evident in experiments in which trace amounts of S287A Cdc25 injected into oocyte cytoplasm were able to induce GVBD, whereas similar amounts of wild-type Cdc25 were not. However, GVBD was only induced in oocytes treated with leptomycin B to inhibit CRM1-mediated nuclear export. A simple hypothesis to explain this requirement would be that oocyte maturation required retention of the imported Cdc25 in the nucleus. We tested this by injecting trace amounts of a doubly mutant Cdc25 lacking both a functional NES and the ability to bind 14-3-3. Although this protein was efficiently imported into and retained in oocyte nuclei, it did not promote GVBD unless oocytes were also treated with leptomycin B (data not shown). This suggests that factors other than Cdc25 must be retained in the nucleus to collaborate with the S287A Cdc25.

It has been reported that 14-3-3 binding to phosphorylated Cdc25 does not greatly affect its activity *in vitro*, producing a <2-fold reduction in activity (Peng *et al.*, 1997; Kumagai *et al.*, 1998b). However, even a slight increase in Cdc25 enzymatic activity resulting from the G_2/M loss of 14-3-3 binding might be sufficient, after concentration in the nucleus, to activate a small amount

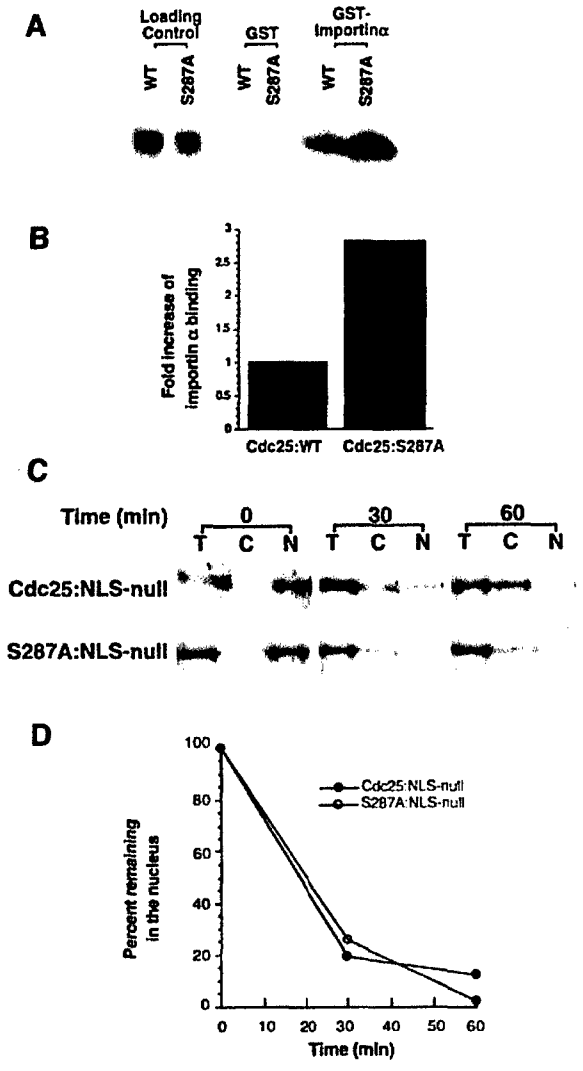

Fig. 9. The Cdc25:S287A mutant binds significantly better than the wild-type protein to importin-α/β, and import-defective variants of these proteins export from nuclei at a similar rate. (**A**) *Xenopus* importin-α protein was fused to GST and coupled to glutathione–Sepharose beads. Twenty microliters of this resin or control GST resin was incubated in 100 μl of interphase egg extract with 10 μl ^{35}S-labeled wild-type or S287A Cdc25 protein for 1 h. The beads were then pelleted and washed five times with egg lysis buffer. The bead-bound proteins were analyzed by SDS–PAGE followed by autoradiography. (**B**) The bar graph represents a quantitation of the data in (A), comparing the amounts of Cdc25:WT or Cdc25:S287A bound to importin-α/β (importin-α beads will associate with importin-β in the extract). (**C**) Cdc25:NLS-null or S287A:NLS-null protein was translated in reticulocyte lysates in the presence of [^{35}S] methionine and injected into *Xenopus* oocyte nuclei. At 0, 30 and 60 min after injection, the oocytes were dissected and successfully injected oocytes were identified by the presence of pink coloring from the reticulocyte lysate. These oocytes were separated into cytoplasmic and nuclear fractions, extracted and analyzed by SDS–PAGE followed by autoradiography. (**D**) The graph represents a quantitation of the data in (C), showing the percentage of Cdc25 remaining in nuclei after 0, 30 and 60 min.

of nuclear Cdc2–cyclin B; this would effectively set in motion a positive feedback loop. In the absence of bound 14-3-3 protein, Cdc25 may also be more susceptible to activation, either by Cdc2–cyclin B (in a feedback loop) or by other nuclear Cdc25-activating kinases. Reconstitution of the Cdc2–cyclin B–Cdc25 positive feedback loop with fully purified components will be required to distinguish between these possibilities. None the less, it is attractive to speculate that Cdc2–cyclin B is the factor which must be concentrated in the nucleus along with Cdc25 to promote passage through the G_2/M transition. Once separated from the cytoplasmic Myt1 (and given the absence of nuclear Wee1 in oocytes), nuclear Cdc25 and Cdc2–cyclin B could very effectively activate each other through positive feedback mechanisms.

Materials and methods

Oocyte preparation, microinjection and subcellular fractionation

Stage VI oocytes of *Xenopus laevis* were prepared for microinjection, dissection and subcellular fractionation as described previously (Yang *et al.*, 1998). Two injection controls for nuclear integrity were used: ^{14}C-labeled bovine serum albumin (BSA; Amersham) and *in vitro*-translated ^{35}S-labeled GRP94 (a protein that does not have an NES or NLS). At each timepoint, five to 10 oocytes were collected for subfractionation and analyzed by SDS–PAGE (National Diagnostics protogel), followed by autoradiography or Western blotting.

Preparation of Xenopus oocyte and egg extracts

Oocyte extracts were prepared as described previously (Shibuya *et al.*, 1992). To induce GVBD, oocytes were treated with 5 μg/ml progesterone in modified Barth's (MB) buffer (Swenson *et al.*, 1989) overnight. Once GVBD was observed in the majority of oocytes, they were collected to make GVBD extracts; these extracts were prepared in mitotic egg extract buffer (Smythe and Newport, 1991) to preserve meiotic phosphorylations. Interphase egg extracts and mitotic extracts were prepared according to the protocols of Smythe and Newport (1991).

Coimmunoprecipitation experiments

Various extracts were incubated with the relevant sera at 4°C for 1 h. Protein A–Sepharose beads (Sigma, St Louis, MO) were then washed with extract buffer and incubated with the above extracts at 4°C for 2 h. Beads were pelleted and washed five times with the relevant extract buffer. Bead-bound proteins were analyzed by SDS–PAGE followed by Western blotting.

In vitro translation

Xenopus Cdc25 wild-type, S287A, NLS-null and NES-null mutants were subcloned downstream of an SP6 promoter in the vector SP64T. ^{35}S-labeled proteins were produced using the SP6-coupled TNT reticulocyte system (Promega) according to the manufacturer's instructions.

Construction of the GST-N-terminal and C-terminal Cdc25 fusion proteins and the S287A Cdc25 mutant

To construct the N-terminal fragment of Cdc25, a stop codon was inserted after amino acid 322 by polymerase chain reaction (PCR) and the resulting clone was inserted into pGexKG through *Nco*I and *Xho*I sites incorporated into the oligonucleotides used for PCR. For production of the C-terminal fragment of Cdc25, amino acids 323–550 of Cdc25 were isolated by PCR and cloned into pGexkG through the *Nco*I and *Xba*I sites.

To produce the S287A mutant clone, a *Xenopus* cDNA clone kindly provided by Dr J.Maller (cdc25C1) was used as the template. The mutant was generated using the method described by Kunkel and colleagues (Kunkel, 1985; Kunkel *et al.*, 1987). Briefly, the mutant primer, 5′CCTTTACCGCTCACCTGCTATGCCAGAGAAAC3′ was annealed to a single-stranded pBluescriptSK$^+$–cdc25C1 DNA and the complementary strand was synthesized *in vitro*.

Construction of the Cdc25 N-terminal truncation series

The Erase-a-Base system (Promega) was used to generate a deletion series from amino acid 322 towards the N-terminus of Cdc25 in pGexKG. The DNA encoding the N-terminal fragment of Cdc25 in pGexKG was cut at its 3′ end with *Xho*I to generate a 5′ overhang for Exonuclease III digestion. It was also digested with *Sac*I to generate an adjacent 3′ overhang to protect the plasmid vector from Exonuclease III digestion. Exonuclease III deletion, ligation and transformation protocols were as

described by the manufacturer. Fifty clones were induced for GST-fusion protein expression. Based on their sizes, five clones were selected for binding assays.

Site-directed mutagenesis

For construction of the NES-null mutant Cdc25, PCR-based mutagenesis was used to mutate both Leu53 and Val55 to Ala. Wild-type *Xenopus* Cdc25 cDNA in the pSP64T vector was used as the template. The 5′ oligonucleotide encoding the N-terminus of Cdc25 was 5′-AATAGTG-AAGCCATGGCAGAGAGTCACATA-3′, where an *Nco*I site was inserted before the start codon. The 3′ oligonucleotide encoding the C-terminus of Cdc25 was 5′-GCGGCGGCCTCGAGATTAAAGCTT-CATCAGGCG-3′, where an *Xho*I site was inserted after the stop codon. The mutagenic PCR primers were 5′-TTGACACCTGTGACTGACGC-TGCAGCTGGATTTAGTAACCTAAGTAC-3′ and its reverse primer. Using the 5′ primer along with one mutagenic primer, we produced a PCR fragment extending from the 5′ end (encoding the extreme N-terminus) to the site of mutation. We then generated a second PCR product using the C-terminal primer and the second mutagenic primer, producing a DNA fragment extending from the mutation site to the C-terminal end. A full-length mutant clone was generated with an additional round of PCR, using a mixture of the N- and C-terminal encoding DNA fragments as templates for PCR with the original 5′ and 3′ primers. The full-length product was subcloned into the pSP64T vector. For construction of the NLS-null mutant Cdc25, the same strategy was used to mutate Lys313, Arg314 and Arg315 to Ala in order to destroy the consensus bipartite NLS. The PCR primers containing mutations were 5′-GAAACACCTGTCAGAGTGGCCGCGGCACGTA-GTACCAGCAGCCCC-3′ and its reverse primer. All mutations were confirmed by DNA sequencing.

Expression and purification of recombinant GST-fusion proteins

All constructs were expressed in Topp 3 *E.coli* (Stratagene). To increase solubility of recombinant proteins, bacteria were grown to OD = 0.5 at 37°C and then shifted to 18°C. Isopropyl β-D-thiogalactoside (0.4 mM) was added to induce protein expression at 18°C overnight. Bacteria were pelleted and resuspended in lysis buffer [50 mM Tris7.5, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. 1% Triton X-100 and 300 mM NaCl were added to increase protein solubility. Cells were lysed twice using a French Press and spun at 17 000 *g* for 30 min. The supernatants were diluted 1:1 with buffer (10 mM HEPES pH 8.0 and 1 mM DTT) to reduce the Triton concentration and incubated with glutathione–Sepharose beads at 4°C for 1–2 h. Beads were pelleted, washed with buffer (10 mM HEPES, pH 8.0, 300 mM NaCl and 1 mM DTT) and kept in storage buffer (10 mM HEPES pH 8.0, 50% glycerol, 1 mM DTT) at –20°C.

Pull-down experiments

GST fusion proteins were coupled to glutathione–Sepharose beads and washed with the appropriate extract buffer. The beads were then incubated with either different extracts or with extracts containing ^{35}S-labeled proteins at 4°C for 1–2 h. The beads were washed five times with the proper extract buffer and the binding proteins were resolved by SDS–PAGE followed by immunoblotting or autoradiography.

Histone H1 kinase assay of single oocytes

Single oocytes were thawed in 20 µl of oocyte lysis buffer (20 mM HEPES 7.5, 80 mM β-glycerolphosphate, 15 mM $MgCl_2$, 20 mM EGTA, 50 mM NaF, 1 mM sodium vanadate, 10 µg/ml aprotin/leupeptin, 1 mM PMSF) and lysed by pipetting. The sample was then microfuged at 4°C for 5 min and the supernatant was collected. To start the kinase reaction, 10 µl of the supernatant was incubated with 10 µl kinase buffer (20 mM HEPES 7.3, 10 mM EGTA, 20 mM $MgCl_2$, 10 µM protein kinase inhibitor (PKI), 0.2 mg/ml histone H1, 0.2 mM ATP and 0.5 µCi/µl [γ-^{32}P]ATP) at room temperature for 10 min. The reaction was stopped by addition of 20 µl of 2× SDS sample buffer and subjected to SDS–PAGE and autoradiography.

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